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THE OCCURRENCE OF GASTRIC LESIONS IN RATS.*

Their Relation to Dietary Deficiency and Hair Ingestion.

By ALWIN M. PAPPENHEIMER, M.D., and LOUISE D. LARIMORE, M.D.
With the Assistance of Robert Solley and Yale Kneeland, Jr.

(From the Department of Pathology of the College of Physicians and Surgeons, Columbia University, New York.

Plates 25 to 28.

(Received for publication, July 11, 1924.)

In examining the gastrointestinal tract of rats which were being used for other experiments, our attention was directed to the frequent occurrence of lesions in the rumen or esophageal portion of the stomach. Since these lesions were found only in rats on variously deficient diets, it seemed interesting to pursue the subject further in the hope of defining the particular factor concerned.

Before describing the lesions encountered, the normal structure of the non-glandular portion of the rat stomach may be briefly recalled.

The rumen of the normal stomach is sharply divided from the glandular portion by a smooth, elevated linear ridge which passes to either side of the cardiac orifice of the esophagus. Elsewhere, the internal surface is uniformly smooth. Histologically, the rumen, including the limiting ridge, is lined with squamous epithelium, surmounted by a thin keratin layer. The superficial cells contain eleidin granules. The thickness of this epithelial layer varies with the state of distention of the stomach. When the organ is contracted, the mucosa is thrown up into low, regular corrugations.

The epithelium rests almost directly upon the well developed muscularis mucosæ, from which it is separated by a delicate connective tissue. This may contain an occasional lymphoid cell or eosinophil, but in the normal organ, this zone is very inconspicuous except at the limiting ridge, where the elevated epithelium is supported by a very considerable amount of rather dense muscular and connective tissue, often quite thickly infiltrated with lymphoid and eosinophil leucocytes.

Between the muscularis mucosæ and the muscularis, there is a well developed layer of a very loose areolar connective tissue, which permits the epithelium to

* A preliminary note referring to this work has recently appeared (Pappenheimer, A. M., and Larimore, L. D., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 141).

adapt itself to the varying states of distention or contraction. This layer normally contains very few wandering cells.

The pathological changes which we wish to describe are situated by preference near the limiting ridge, and most frequently near the lesser curvature and the esophageal orifice. They may, however, involve other portions of the rumen. They appear as more or less elevated round or oval discs, with a small central, ulcerated brownish depression (Fig. 1). The lesions are either discrete or more or less confluent. In the more severe or advanced cases, the lining of the rumen is thrown up into papillomatous or cauliflower-like growths, accompanied often by a marked hypertrophy of the muscular coat. The demarcating ridge is often involved, presenting a nodose, beaded appearance (Fig. 2).

These alterations are found, microscopically, to involve the entire wall of the stomach.

The epithelium frequently shows a marked thickening and exfoliation of the keratin layer, particularly near the opening of the esophagus. The epithelium is increased in thickness; blunt processes extend down to the muscularis mucosæ, or the epithelium is reduplicated to form papillary projections into the lumen. Mitotic figures are often numerous in these hyperplastic areas.

The actively proliferating epithelium rarely penetrates the muscularis mucosæ, but occasionally irregular solid processes in continuity with the surface epithelium, extend into the submucosa. In one instance, there was a large cystic evagination (Fig. 6).

Ulceration and necrosis, usually superficial, but sometimes extending into the submucosa, is a frequent, but not invariable finding. The ulcers are surmounted by debris of keratin flakes, dead epithelial cells, and leucocytic fragments, Bacteria and yeasts are abundant on the surface, but do not extend deeply into the tissues. Repeated search for mycelium in Gram and Giemsa stained sections was negative.

Accompanying these changes in the epithelium, the subjacent connective tissue, both above and below the muscularis mucosæ, shows edema, often fibrinous, and a more or less intense cellular infiltration. The predominant type of wandering cell was the polymorphonuclear eosinophil, which is present normally in considerable numbers in the stroma of the glandular mucosa, but is scarce and inconspicuous in the esophageal portion of the rat stomach. Near the ulcerated foci, polymorphonuclear neutrophils predominated. Lymphocytes, and larger mononuclear elements were also abundant. The capillaries and veins in the inflamed area were wide and congested. In the less acute areas, the edematous submucosa contained numerous proliferating fibroblasts and new forming blood vessels (Figs. 3, 4, and 5).

Influence of Diet on Incidence of Lesions.

Rats on Complete Diet.—On a stock diet, consisting of whole grain, greens and meat, with or without the addition of milk, no lesions other than a mild keratosis were observed in any of the nineteen rats examined. Fifteen wild grey rats, caught in the laboratory—all apparently well nourished and healthy—showed normal stomachs. The findings in rats on complete artificial diets which served as controls to the experiments were, with occasional exceptions, to be discussed later, negative. It has been our experience, therefore, that the lesions occur only on artificial and deficient diets.

Rats on Diet 84.—The highest incidence of the lesions was found in the thirty-five rats which belonged to this group. The diet is the one used in this laboratory for the experimental production of rickets, and consists of patent flour 95.0, calcium lactate 2.9, sodium chloride 2.0, and ferric citrate 0.1. As has been pointed out in previous papers (1) this diet is deficient in the character of its proteins, in its vitamine A content, in various mineral constituents, notably phosphate, and possibly to a limited degree in vitamine B. Rats placed on the diet at the age of 4 weeks show only a limited growth, and unless treated with cod liver oil or ultra-violet radiations, become rachitic.

Of thirty-five young rats on this diet for periods of from 27 to 35 days, thirty-one or about 86 per cent, showed gastric lesions of greater or lesser severity. Definite ulcers were present in the majority, and since complete serial sections were not made, it is probable that they were overlooked in some of the rats.

An interesting point is brought out by this series. Six of the rats received a daily exposure to the rays of the quartz mercury lamp for 15 minutes at a distance of 9 feet. This treatment, sufficient to protect against the development of rickets, did not prevent the gastric lesions.

In addition to the thirty-five rats on Diet 84, we examined the stomachs of ten rats on diets in which the mineral constituents of Diet 84 had been altered in various ways; in other respects, these diets presented the same deficiencies as Diet 84. Six of these rats showed definite ulcers, and in all but one of the others, there were more or less striking epithelial proliferation and submucous edema. All of the

three rats on Diet 85C, which is low in calcium, but contains an excess of phosphates, showed distinct lesions. A deficiency of phosphate is therefore not a factor in their production.

Experiments on the Possible Influence of Excess of Base or Acid.

It seemed possible that the excessive alkalinity of Diet 84, due to its high calcium lactate content, might be an important factor, either because of its directly injurious effect upon the epithelial lining cells, or conceivably, by altering the reaction of the gastric contents so as to favor bacterial growth.

It was decided to investigate this possibility experimentally by placing rats upon diets nutritionally complete, but rendered acid or alkaline by the use of salt mixtures containing excess of sodium or chlorine ions respectively.

The first two experiments of this sort, in which eighteen rats were used, were negative. Normal stomachs were found in every case, in spite of the abnormal and unbalanced mineral content of the diet. It seemed improbable, as a result of these preliminary observations, that the alkalinity or acidity of the diet *per se* could be a factor in the production of the lesions. A third experiment, however, gave different results, and may be briefly discussed.

The composition of the salts in the diets is given in Tables I and II. Of the four rats on the alkaline diet, two showed multiple ulcers with marked epithelial hyperplasia; two had normal stomachs. The four rats on the acid diet showed more or less epithelial hyperplasia about the marginal ridge, but no ulcers, or acute inflammatory changes.

We have no explanation to offer for the discrepancy between this experiment and the preceding ones. We shall consider later the evidence in favor of the possibility that a deficiency in vitamine A or some related accessory food factor predisposed to the development of the gastric lesions. In making up the diets for the alkali and acid experiments, it was assumed that the unextracted commercial casein used contained an adequate amount of vitamine A. This may, however, not have been the case, and since two different samples of casein were used in the preparation of the diets, the possibility cannot be excluded that the second sample contained less vitamine A than the first.

The occurrence of a mild xerophthalmia in several of the rats as well as their subnormal growth, suggests that there may have been a relative insufficiency.

TABLE I.

Composition of Alkaline and Acid Diets.

Alkaline Diet.

			H_2O
Alkaline salt II.	KCl	0.850	
	$MgCO_3$	0.207	
	Ca lactate	4.083	1.192
	$Na_2HPO_4 \cdot 12\ H_2O$	4.738	2.857
	Ferric citrate	0.100	
	Traces mixture	0.0037	
Total		9.982	4.049
Total per cent of salt − water of crystallization = 5.933			

Acid Diet.

			H_2O
Acid salt II.	$CaCl_2$	0.735	
	KCl	0.850	
	NaCl	0.850	
	$CaH_4(PO_4)_2 \cdot H_2O$	1.668	0.119
	NH_4Cl	1.600	
	$MgSO_4 \cdot 7H_2O$	0.507	0.239
	Ferric citrate	0.100	
	Traces mixture	0.0037	
Total		6.314	0.358
Total per cent of salt − water of crystallization = 5.956			

TABLE II.

Calculated Mineral Content of Diets.

	Na	K	Ca	Mg	P	Cl
Alkaline	0.608	0.445	0.530	0.059	0.410	0.405
Acid	0.334	0.445	0.530	0.050	0.410	2.446

Experiments on the Possible Relation of the Gastric Lesions to Deficiency in Fat-Soluble Vitamine.

In addition to the thirty-five rats on Diet 84, referred to above, our material included seven rats on this diet, which had received treatment with cod liver oil for periods of from 12 to 22 days, after having been on Diet 84 for 4 weeks. Gastric lesions were present in but two of these, and in one the ulcers appeared to be healing. This smaller incidence suggested a further inquiry into the protective action of the cod liver oil, and the possible rôle of a deficiency of vitamine A, or related food factors in the causation of the lesions.

The stock diet used in these experiments consisted of:

Casein (inactivated by heating 24 to 48 hrs. at 105° in shallow pans)	21.0
Duryea's starch	57.7
Crisco	17.0
Yeast (Harris' concentrated extract)	0.6
Salt Mixture 185 (McCollum)	3.7

The usual precautions were taken in regard to the care of the animals. Ash-free filter paper was given for roughage. A record was kept of the daily food consumption. Bergen cod liver oil, in some of the experiments, was administered by pipette to the amount of 0.1 to 2.0 cc. daily; in other experiments, it was incorporated in the diet to the amount of 2 per cent, replacing an equivalent weight of Crisco.

An analysis of our data brings out several significant facts. Of twenty rats maintained for periods varying from 9 to 97 days on a diet in which the only known deficiency was a lack of vitamine A, gastric lesions were present in eleven or 55 per cent. Four rats, showing only slight epithelial overgrowth and hyperkeratosis, without inflammatory lesions, are included among the negatives.

Although it seems evident that the lack of the fat-soluble factor predisposes in some way to the development of the lesions, it is equally clear that there is no constant relation between the occurrence of the lesions, or their severity, and the length of time that the animal was on the deficient diet. Thus Rat 80–00, after only 21 days, showed severe hyperplastic and ulcerative lesions, whereas rat 80–09, after 97 days, showed only a slight epithelial hyperplasia, with no inflammatory or ulcerative lesions.

A second fact disclosed in these experiments is that the addition of cod liver oil in every instance protected completely against the development of the gastric lesions. Normal stomachs were found in eleven rats in which cod liver oil was added to or included in the vitamine A deficient diet.

Included are seven rats which received 2 per cent of cod liver oil, inactivated on a water bath at the temperature of boiling water for 20 hours in the presence of an air current. The experiments of Hopkins (2), of Drummond and Coward (3), and of McCollum (4), have shown the destructive effect of heat and oxidation upon the antixerophthalmic potency of butter and cod liver oil. In accord with their observations, xerophthalmia developed with great rapidity and severity in all seven rats given the inactivated oil. In spite of the loss of its antixerophthalmic property, there was, in this small series, complete

TABLE III.

Diet.	No. of rats.	No. of rats showing lesions.
84	5	2
84 plus 2 per cent cod liver oil (inactivated).	6	3
84 " 2 " " " " " (whole).	6	3

protection against the gastric lesions. This strongly suggested that the protective effect was not due to the vitamine A content of the cod liver oil—or more specifically, to its antixerophthalmic factor.

If the absence of lesions in the foregoing experiment is to be attributed to a specific substance or substances contained in the cod liver oil, this protection should be manifest even when the animals are placed upon a highly deficient diet such as Diet 84.

The following experiments, however, showed that neither the whole cod liver oil, nor the inactivated oil were capable of preventing the gastric lesions, when added to Diet 84 to the amount of 2 per cent.

Two litters were used for the experiment, and it was noted that whereas in one litter, only one of nine showed lesions; in the other, seven out of eight were affected. The two litters were of approximately the same age and weight and were obtained from the same source.

Although the cod liver oil, when incorporated with the diet, failed to protect, the experiment was repeated on two other litters, the oil being administered separately by pipette, to the amount of 2 or 3 drops daily over a period of 28 days.

From these experiments, it became evident that other factors were concerned in the production of the lesions than the absence of specific substances in the cod liver oil, and indeed, it seemed highly probable that vitamine deficiency *per se* played no part in their causation.

It seemed possible, on further reflection, that the deficient diets, regardless of the particular deficiency concerned, might result in a loss of gastric motility, and that stasis of the gastric contents might be at least a predisposing cause for the development of the lesions. But this hypothesis also did not survive an experimental test.

TABLE IV.

Diet.	No. of rats.	No. of rats showing lesions.
84	5	5
84 plus 2 to 3 drops inactivated cod liver oil.	5	4
84 " 2 " 3 " of whole cod liver oil.	6	3

Barium sulfate and distilled water were added to Diet 84, making a thin paste which could be introduced without difficulty into the stomach through a tube. The motility was studied fluoroscopically, observations being made at frequent short intervals and the emptying time recorded. The observations on most of the rats were repeated after a few days or a week, and it was found that the emptying time for the individual rat remained quite constant. There were, however, wide individual variations ranging from 10 minutes, in the case of one rat, to several hours. There proved to be no correlation between the occurrence of the lesions and the emptying time of the stomach during life. Indeed, the rat with the shortest emptying time was found subsequently to have most severe ulcerative and papillary lesions. It is unnecessary to present these data in detail, since they throw no light upon the pathogenesis of the lesions.

The Ingestion of Hair as a Possible Factor.

We had made the observation that the stomachs of rats on deficient diets frequently contained hair in considerable amount, but had attributed no particular importance to the finding until we chanced to discover, in the sections of several of the rats, portions of the hair shaft embedded in the tissue. Thus in Rat 80–97 (Fig. 6), there was a large cystic invagination of the epithelium, extending through the muscularis mucosæ to the muscularis. Amongst the exfoliated keratin flakes forming the contents of the cyst, was found a long hair shaft.

In other sections from the same rat, a fragment of hair was found in the submucosa, surrounded by inflammatory tissue, with numerous foreign body giant cells.

In Rat 80–56, which had been for 33 days on a minus A diet, a splinter of hair was found embedded in the hypertrophied epithelium adjacent to an ulcer. The epithelium contiguous to the hair fragment showed pycnosis and shrinkage of the nuclei.

In several other sections also, refractile splinters were seen amongst the epithelial cells, or amongst the inflammatory cells in the submucous tissue. Although these could not be positively recognized as portions of hair shafts, the definite observations mentioned above suggested a new possibility, namely that hair ingested by rats on a deficient diet might traumatize the mucosa sufficiently to produce the lesions. The punched out character of the ulcers was in favor of such a possibility; indeed, on close study, many of the sections showed sharp breaks in the surface epithelium such as might have resulted from puncture by a rigid hair shaft.

It seemed worth while to attempt to reproduce the lesions by incorporating a large amount of hair in the diet. A diet was prepared consisting of:

Casein	21.0
Starch	57.7
Salt 185	3.7
Yeast extract	0.6
Crisco	14.0
Cod liver oil	3.0

The Crisco was melted, and sufficient finely cut rat hair added to make a thick paste. This was then intimately mixed with the remain-

ing constituents of the diet. That the hairs were actually ingested with the food was proven by finding them abundantly in the feces. Red blood cells were also present. Six young rats were then placed on the hair diet, and six controls of the same litter on the same diet without the addition of hair.

In the six control rats, killed at intervals of 4 to 6 weeks, the stomach was found normal in all but one, and this showed merely a very slight papillary hyperplasia, over a small area along the greater curvature. Of the six rats on the hair-containing diet, one (No. 90–35) showed three ulcers in the non-glandular portion; with the hand lens several hairs could be seen sticking into the central punched out crater. Another rat (No. 90–36) presented an area of papillomatous thickening along the lesser curvature, with several hairs embedded in the mucosa. The remaining four rats were free from gross lesions.

Serial sections of the ulcers in Rat 90–35 (Fig. 7) showed the hair fragments penetrating deep into the mucosa, and surrounded by an inflammatory reaction.

DISCUSSION.

The most interesting and best established fact revealed by these data is that the incidence of the gastric lesions is definitely related to dietary deficiency. This is well brought out if we compare the occurrence of the lesions in the entire series of rats on deficient and complete diets respectively. In the first group, comprising in all 112 rats, lesions were encountered in 68—an incidence of approximately 61 per cent. In the second group, which includes animals on stock diet,[1] on a supposedly complete synthetic diet rendered acid or alkaline by varying the mineral content, and on a fat-soluble vitamine deficient diet completed by the addition of cod liver oil, gastric lesions were found in but five out of 66 rats, or approximately 7.0 per cent. In the case of three of these five rats, there was a possibility that the casein used was inadequate in regard to its vitamine A content. In any case, the difference in the incidence in the two groups of animals is too great to attribute to chance.

From a study of the literature, we find that similar proliferative and ulcerative lesions of the proventriculus of rats have been observed by others, and quite variously interpreted.

[1] The fifteen wild rats are also included.

Mention should first be made of the widely known and exhaustive studies of Fibiger. His researches on the papillomatous and carcinomatous tumors of the rat stomach and their relation to infestation with nematode *Spiroptera neoplastica* (*Gongylonema neoplasticum*) are too familiar to require further citation. A comparison of the earlier lesions illustrated in the beautiful plates which accompany Fibiger's first larger publication (5) with the lesions described by us, show a minute correspondence in the gross and microscopic features. A careful study of our preparations, however, and repeated examination of stomach contents, and in some cases, of the feces for nematode ova, has failed to show infestation with *Gongylonema* in our animals. Although we have had no personal opportunity to examine Fibiger's preparations, the pictures of the worm in his plates are so distinct that there should be no difficulty in identifying it, were it present. In a number of our rats, serial sections were cut through the ulcerated areas in the hope of discovering a parasite, but always without success. We feel quite certain that the lesions described by us, in spite of their morphological identity with the non-cancerous stages described by Fibiger, are not associated with nematode infection.

We have found but one reference in the articles of Fibiger to the diet of his experimental animals. In an article published in 1920 (6), it is stated that the rats were fed exclusively on white bread. Whether this inadequate diet favored the development of the lesions apart from the presence of the parasites, cannot be decided.

Quite similar lesions also were found by Rohdenburgh and Bullock to follow the introduction of spinous objects into the stomach of rats (7). The authors, however, expressed themselves with some reserve as to the rôle played by the mechanical irritant, since they had observed similar lesions in non-operated rats. No data are given in the articles as to the dietary conditions of these animals, but we have been informed by Dr. Rohdenburgh that the rats received bread, with turnips, carrots, or greens once or twice weekly. The possibility of a dietary deficiency in these animals can hardly be positively excluded.

Buschke and Lange (8) describe tumor-like changes in the mucosa of the prostomach of rats as a result of tar, injected *per rectum* in doses of 0.1 to 0.3 cc. twice weekly. Of 54 animals thus treated, all but four showed lesions similar to or identical with those described by us. No lesions were found in the rectum, nor in the remaining portion of the intestinal tract, and no cancerous transformation occurred in any of the animals.

As controls, the authors refer to another series of rats used for bacteriological purposes, to a series injected with paraffin or Scharlach R in oil, and to Fibiger's negative controls. In all these instances, normal stomachs were found.

It does not appear, however, that the authors examined rats of the same litters, kept under identical conditions as regards diet. Indeed, no mention is made of the food.

Buschke and Peiser (9) describe identical lesions in the rat stomach, this time provoked by the oral or subcutaneous administration of 0.2 mg. of thallium.

About 80 per cent of the animals—that is, about the same proportion as the tar rats—were affected. Again no reference is made to diet.

The interpretation of our findings is more difficult than their description. Deficient diets indubitably favor the occurrence of the lesions, and there can be no question that in experiments such as those of Buschke and Lange, a failure to take the dietary influence under consideration weakens the validity of their conclusions.

But as to the precise nature of this dietary influence, we can as yet say nothing definite. Our experimental efforts to analyze the factors in terms of known dietary deficiencies have proven inconclusive, and we are led to believe that there is another factor involved.

While our work was in progress, and shortly after we had independently observed the occurrence of hair or hair fragments in the lesions, there appeared the interesting paper of Mitchell, Bradshaw, and Carlson (10). These writers describe the finding of definitely formed hair-balls in the stomach of 90 per cent of rats kept on the standard casein paste diet of Osborne and Mendel. They were never seen in rats on stock diet of mixed foods, on dry, crumbly diets, or on liquid diets.

They say further that the constant irritation of the hair-balls may cause inflammation of the gastric mucosa, and in several cases, microscopic examination disclosed a definitely ulcerated condition. The lesions are not described in detail, and it is not stated whether they affected the glandular or esophageal portion of the stomach.

Our experience differs from theirs in that we found ingested hair, though not well formed hair-balls, on the dry diets used in our experiments, Diet 84 for example. The high fat content of the diet cannot be at fault in these cases.

CONCLUSIONS.

Ulcerative lesions of the rumen with papillary epithelial hyperplasia occur in a large proportion of rats maintained on a deficient diet, and are rarely, if ever, present when the diet is complete.

It has not been possible to ascribe the lesions to lack of any of the known dietary components.

The fact that rats on improper diets tend to eat hair, that hair fragments have been found in a number of instances embedded in the

ulcers, and surrounded by a definite inflammatory reaction, and finally, that the ulcers have been experimentally produced in rats on a *complete* diet, when chopped hair was added to the food, lead us to believe that the ingested hair is an important factor in the causation of the lesions. Whether the hair splinters act merely as mechanical irritants, or as the carriers of an infective agent, has not been determined. It is probable, moreover, that the deficient diets in some way that we do not as yet understand, intensify the injury caused by the hair. However produced, the lesions are so frequently found that they should be taken into account in experimental work on rats, especially in judging of the effects of dietary influences by the weight curves. That the presence of severe ulcerative lesions in the prostomach must unfavorably affect the general nutrition of an experimental animal, seems beyond question.

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EXPLANATION OF PLATES.

PLATE 25.

FIG. 1. Rat 80–51. 46 days on alkaline diet. Large ulcers, in places confluent, with marked hyperplasia of adjacent mucosa.

FIG. 2. Rat 60–97. Diet 84, 30 days. Numerous ulcers and extreme papillary hyperplasia affecting the entire mucosa of the rumen. Note the beaded thickening of the limiting ridge.

PLATE 26.

FIG. 3. Rat ?. Hyperkeratosis, marked papillary hyperplasia of epithelium, edema and inflammatory infiltration of submucosa. Superficial central ulcer, covered by yeasts and bacteria.

PLATE 27.

FIG. 4. Rat 22–96. Diet 84, 47 days. Marked papillomatous hyperplasia. *K*, thickened keratin layer. *ep*, proliferated epithelium. *mm*, hypertrophied muscularis mucosæ. *sm*, edematous submucosa. m_1, m_2, circular and longitudinal muscle fibers. *inf*, inflammatory infiltration of subepithelial connective tissue.

FIG. 5. Rat 80–19. 53 days on vitamine A deficient diet. Section through inflamed and thickened gastric wall. *u*, superficial area of ulceration. *e*, papillary down-growth of epithelium. *c*, clefts in submucosa which probably contained hair fragments.

PLATE 28.

FIG. 6. Rat 80–97. 29 days on Diet 84 plus 2 drops of inactivated cod liver oil daily. The section shows a large cyst-like invagination (*c*) of the gastric mucosa. It is filled with loose keratin flakes, and contains also a portion of a *hair shaft* (*h*). The superficial epithelium shows marked papillary hyperplasia. There is a small ulcer, with inflammatory reaction at *u*. *cm*, circular muscle. *lm*, longitudinal muscle.

FIG. 7. Rat 90–35. 29 days on complete synthetic diet with admixture of rat hair. Section showing hair shaft embedded in epithelium and submucosa. Note ulceration and local inflammatory reaction.

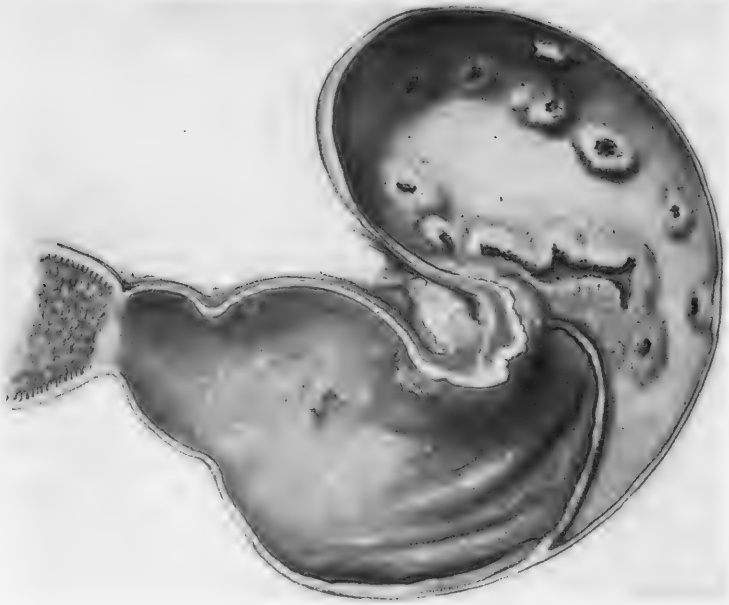

FIG. 1.

FIG. 2.

(Pappenheimer and Larimore: Gastric lesions in rats.)

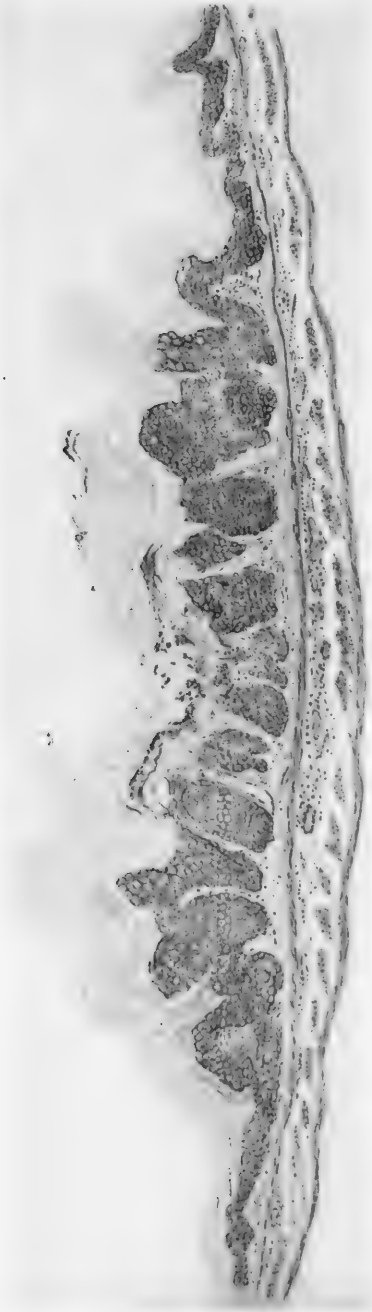

FIG. 3.

(Pappenheimer and Larimore: Gastric lesions in rats.)

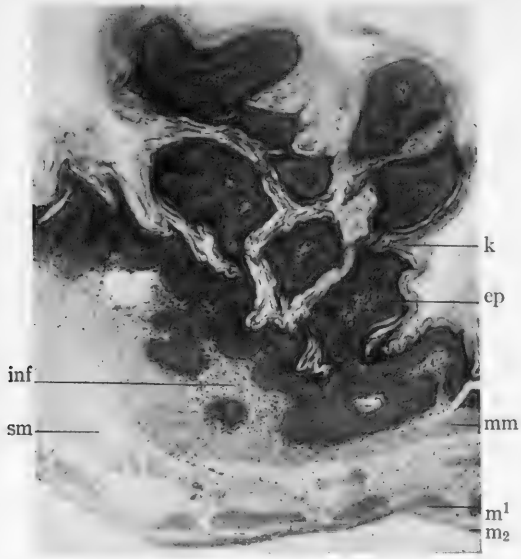

FIG. 4.

FIG. 5.

(Pappenheimer and Larimore: Gastric lesions in rats.)

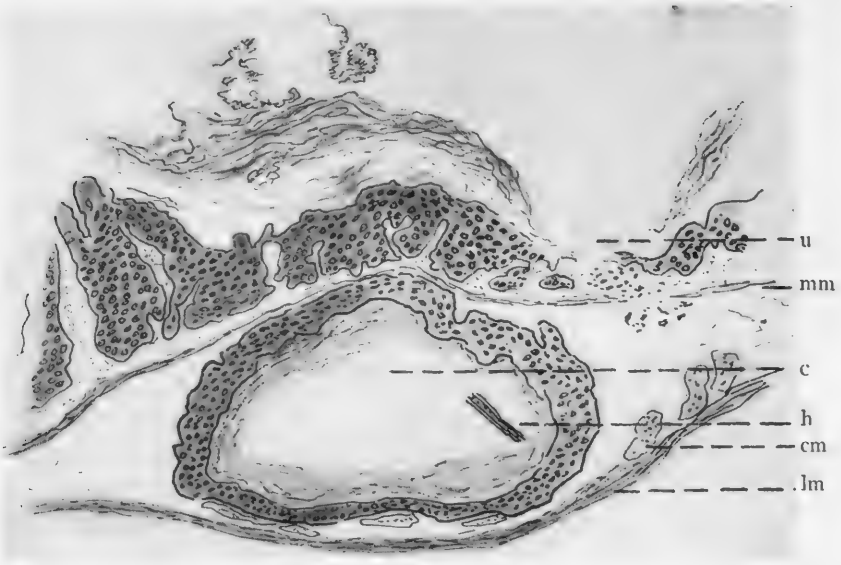

FIG. 6.

FIG. 7.

(Pappenheimer and Larimore: Gastric lesions in rats.)

EXPERIMENTAL PRODUCTION OF PNEUMOCOCCUS PNEUMONIA IN MICE BY THE INHALATION METHOD.

By ERNEST G. STILLMAN, M.D., and ARNOLD BRANCH, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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Many attempts have been made to produce pneumonia in animals with the pneumococcus since Fraenkel (1) and Weichselbaum (2) described this organism as the etiological agent of croupous pneumonia. It early became evident that there were other factors concerned in the production of the disease besides the introduction of the organisms into the respiratory tract. The instances in which experimental lobar consolidation of the lung has been successfully produced by this means are open to the criticism that large amounts of culture material were injected into the respiratory tract under more or less artificial conditions, and that in some instances additional gross injury to the respiratory epithelium was resorted to in order to render the experimental animal susceptible to infection. The experiments here to be reported were planned to obviate as far as possible these objections.

In preceding papers (3) it has been shown that virulent pneumococci generally disappear from the lungs of normal mice within a few hours following inhalation and rarely give rise to a generalized infection. In mice intoxicated with alcohol, however, pneumococci persist in the lungs for a longer period and a fatal septicemia frequently follows. It has further been shown (4) that a high degree of immunity is produced in mice following repeated inhalations of living pneumococci and that resistance is only slightly increased in these animals by repeated exposures to a spray of dead organisms.

It seemed possible that localization of the infection in the lungs might be effected by allowing partially immunized mice to inhale virulent pneumococci while under the influence of alcohol, and the experiments were planned with this in view. We shall here report upon

pulmonary lesions encountered (1) in normal mice which were killed or died while under the influence of alcohol, (2) in normal mice which were alcoholized and allowed to inhale pneumococci while intoxicated, and (3) in partially immune mice which were exposed to an atmosphere of the pneumococci while intoxicated. In this latter group of partially immune mice are included only animals which were immunized by the inhalation method and they fall into three groups: (*a*) mice which had recovered from several exposures to an atmosphere containing living pneumococci, (*b*) mice which had in like manner been exposed to atmospheres containing heat killed pneumococci, and (*c*) mice which had survived, while intoxicated, one or two previous exposures to atmospheres in which living pneumococci were present. There are also included experiments in which a different type of pneumococcus was used for immunization than that employed in the final exposure.

In the course of the study no percentage figures on the incidence of pneumonia produced by the inhalation method were obtained since only animals which died were examined, and it is conceivable that others became infected but recovered.

EXPERIMENTAL.

Method.—The organisms used were virulent strains of Pneumococcus Type I and Type II and a slightly virulent strain of an atypical Type IIc. The latter strain was used instead of a Group IV pneumococcus as the atypical Type II strains belong in the group of organisms which are encountered frequently in normal mouths but which rarely incite disease in man.

The mice to be immunized were placed in the spray chamber previously described (3) and the air inside was repeatedly sprayed at intervals of 2 to 3 days with 50 cc. of either (1) broth culture of virulent pneumococci, (2) broth cultures of pneumococci which had been killed by heating to 60° for 30 minutes, or (3) killed pneumococci suspended in salt solution.

The mice were alcoholized by intraperitoneal injection of 1.5 cc. of a 10 per cent solution of alcohol in saline. 1 hour after the administration of the alcohol the animals were sprayed with 50 cc. of the pneumococcus culture and allowed to remain in the spray box for 1 hour. In the case of death of the animals, cultures were made in broth from the lungs and from the heart's blood. At least one lobe of the lung was fixed in Zenker's fluid for histological study.

Histological Examination of the Lungs of Normal Mice after Alcohol Administration.

In an accompanying paper (5) the pulmonary lesions most commonly found in normal mice, killed by cranial compression, are described in detail. Suffice it to say here that congestion and a very occasional spontaneous pneumonia of predominating mononuclear type were the only lesions noted. In a series of mice which died of acute alcohol poisoning or were killed within a period of 2 hours following the administration of alcohol the lungs were examined microscopically. Congestion was present but no more frequently than in the normal controls and, in fact, no other lesions were observed which did not also occur in the normals. It is necessary to bear this in mind in order to interpret the lesions to be described below as occurring in the lungs of mice which were allowed to inhale pneumococci.

Infection by the Inhalation Method.

The pulmonary lesions encountered in these animals ranged from a simple congestion to the outspoken lesions of a serofibrinous pleurisy, interstitial inflammation, and red and gray hepatization. A detailed discussion of the pathological changes will be given in an accompanying paper (5). For present purposes the interstitial inflammation and red and gray hepatization alone will be considered as affording evidence of localization of the infection in the lungs.

1. Pulmonary Lesions in Non-Immune Mice.—Of a total of 246 normal mice which were alcoholized and exposed in an atmosphere containing virulent Type I pneumococci, forty-one or 16 per cent died of pneumococcus septicemia. The lungs of thirty-three of these forty-one mice were examined histologically. In only one of these were the organs normal. Congestion occurred in nineteen, congestion and serofibrinous pleurisy in eleven, and interstitial inflammation in two, in one of which pleurisy was also present.

In this experiment only two of thirty-three non-immune mice which died of pneumococcus septicemia following inhalations of Type I pneumococci while intoxicated showed any evidence of localization of the infection in the lungs. In these two instances there was an early interstitial inflammation but neither red nor gray hepatization

occurred. On the other hand, a serofibrinous pleurisy associated with the presence of pneumococcus was noted in eleven instances without demonstrable intrapulmonary lesion in the lobe examined.

2. Pulmonary Lesions in Immunized Mice.—In order to determine the effect of similar treatment in partially immunized animals, mice which had survived one exposure to pneumococci while alcoholized were subsequently treated with alcohol and allowed to inhale an atmosphere of pneumococci. Of the 163 mice so treated, eighteen, or 11 per cent died of a pneumococcus septicemia. Histological examination showed the lungs to be normal in three instances; congestion occurred nine times, pleurisy and congestion three times, interstitial inflammation twice, red hepatization once. In other words, fifteen or 83 per cent of the fatal cases showed no evidence of attempts at localization, while three or 16 per cent showed definite localized pulmonary lesions. In one instance the lesion had advanced to the stage of red hepatization.

With a view of demonstrating whether a greater percentage of pulmonary localization would occur in more highly immunized mice, seventy-eight mice which had survived two exposures to a pneumococcus spray while intoxicated were again alcoholized after a period of 10 days and exposed a third time to virulent Type I pneumococci. Seven of these mice died with pneumococcus septicemia. Histological examination of the lungs of these animals revealed congestion in two instances, interstitial inflammation associated with pleurisy occurred once, red hepatization once, and gray hepatization in three instances. In this more highly immune series five, or 71 per cent of the fatal cases, showed marked local reaction in the lungs, as contrasted with 16 per cent in the less immune animals mentioned above.

In a further demonstration of the point that a greater percentage of pulmonary localization occurs in partially immunized than in normal mice, yet another series of 250 mice which had been immunized by spraying from two to ten times with living or heat-killed cultures of pneumococci were alcoholized and exposed to an atmosphere of virulent pneumococci. In each instance, the final exposure was made 10 days after the last "immunizing" spray. Of these 250 partially immunized mice, forty-five or 18 per cent died of pneumococcus septicemia. The lungs of the forty-one mice which were examined his-

tologically were normal in three instances, congestion and pleurisy occurred in nineteen, interstitial inflammation in nine, and red or gray consolidation in ten.

From these experiments it would appear that normal mice infected with pneumococci by the inhalation method die of an overwhelming septicemia without marked cellular reaction in the lungs, whereas partially immune mice which are similarly infected by inhaling pneumococci while alcoholized present in their lungs at death definite inflammatory reactions. It also seems evident that as the mice become more immune there is a progressive decrease in the number of deaths from pneumococcus septicemia without pulmonary localization, and a reciprocal percentage of the fatalities shows pulmonary localization.

3. Pulmonary Lesions in Mice Immunized with One Type of Pneumococcus and Infected by the Respiratory Route by Organisms of a Different Type.—In order to ascertain whether the immunity acquired by the inhalation method is type specific, mice which had been previously immunized by this procedure were later exposed to an infecting spray of virulent organisms of a heterologous type. Since pulmonary localization of an infection occurring by way of the respiratory tract is an evident expression of the degree of immunity acquired by previous exposure, the extent and nature of the local reactions in the lungs were utilized as criteria in these cross-infection experiments.

Nineteen mice were sprayed eight to ten times with a slightly virulent atypical Type IIc, and thirty-two mice were sprayed with the same organism while intoxicated. After a 10 day interval these 51 mice were intoxicated and sprayed with a Type I pneumococcus culture. Nine or 17 per cent of these animals died. Upon histological examination of the lungs two showed congestion, one congestion and pleurisy, two interstitial inflammation, three red hepatization, and one gray hepatization. Of eighteen mice similarly immunized by spraying eight times with Type IIc pneumococcus and subsequently sprayed while alcoholized with virulent Type II pneumococci, six or 33 per cent died. Histological examination of the lungs showed congestion and pleurisy in two instances, interstitial inflammation occurred in two, and in two red hepatization. A total of fifteen of the

69 mice immunized with an atypical Type IIc pneumococcus and exposed to a spray of Type I or Type II pneumococci died. In ten or 66 per cent death was associated with definite pulmonary localization.

In order to see whether mice which had been rendered so immune to Type I pneumococcus that they were no longer amenable to infection by inhalation of the homologous organism, could be infected with a Type II organism, 94 Type I immune mice were again alcoholized and sprayed with a Type II pneumococcus. Histological examination of the lungs of the fifteen mice which died showed them to be normal in one instance, congestion and pleurisy occurred in four, interstitial inflammation was present in two, red hepatization in four, and gray hepatization in four. Thirteen other mice which were so highly immunized against a Type I pneumococcus that they survived an otherwise fatal intraperitoneal injection of 0.000,001 cc. of the same virulent culture were alcoholized and sprayed with Type II pneumococcus. Four of these thirteen more highly immune mice died with septicemia. The lungs of the animals showed, respectively, congestion, congestion and pleurisy, interstitial inflammation, and gray hepatization.

From these experiments it is evident that mice which have been immunized against one type of pneumococcus may show an increased resistance locally when infected by the inhalation method by organisms of another type and, further, that even if they acquire a relatively high degree of immunity to one type of pneumococcus, they may be infected by inhalation with a different type.

The results are summarized in Table I which gives the percentage incidence of the lesions produced in the total 246 non-immunized and 667 immunized mice. Of the thirty-three non-immunized mice which died following exposure to pneumococcus atmosphere while alcoholized, only two showed definite intrapulmonary changes, having the nature of an early interstitial inflammation. On the other hand, in the case of the 100 partially immunized mice which died, definite lobar consolidation resembling the red and the gray stages of lobar pneumonia as seen in man were encountered in thirty instances, the early pulmonary lesion termed interstitial inflammation was found twenty-one times, in six instances associated with a pleurisy, and a

serofibrinous pleurisy occurred thirty-one times without evidence of intrapulmonary inflammation.

It is interesting to note that the localizing pulmonary lesions occurred in only 38 per cent of the mice infected with the same type of pneumococcus as that used for immunization, as contrasted with 64 per cent of localizing lesions in mice infected with a different type of organism from that used for immunization.

TABLE I.

Comparative Incidence of Pathological Lesions in Lungs of Non-Immune and Immune Mice Succumbing to Infection by Inhalation Method.

Lesions.	33 non-immunized mice.	100 immunized by the inhalation method.
	per cent	*per cent*
Normal	3	7
Congestion	58	14
Congestion and pleurisy	33	30
Interstitial inflammation	6	19
Consolidation: red and gray hepatization	0	30

DISCUSSION.

A number of workers (6) have succeeded in producing pneumonia in animals by intratracheal injections. In most instances it has been necessary to instill considerable amounts of culture well down into the trachea before positive results were obtained. As early as 1904, Wadsworth (7) was able occasionally to produce pneumonia in partially immunized rabbits by the injection of 1 cc. of pneumococci into the trachea. The non-immunized rabbits, on the other hand, either remained well or died of septicemia. More recently Jones (8) has found that the introduction of a virulent culture of *B. avisepticus* into the trachea of normal rabbits was sufficient to cause a rapidly fatal septicemia. However, if the resistance of the rabbits had been stimulated by subcutaneous vaccination, instead of the rapidly fatal septicemia without lung involvement, consolidations of the more dependent lobes occurred with considerable regularity.

In a previous paper of this series it has been shown that when mice are exposed to an atmosphere containing virulent pneumococci the organisms may be recovered for a period of 3 hours thereafter by culturing small pieces of the periphery of the lung, but that they are too few to be demonstrated histologically. A generalized infection rarely

occurs in such animals, yet the survivors develop a specific general immunity. As previously pointed out, this suggests that they are not removed merely mechanically from the respiratory tract, but that a sublethal dose of organisms must actually gain access to the tissues and be destroyed. In contrast, when mice are intoxicated with alcohol and sprayed with pneumococci the organisms persist in the lungs for a longer period and a fatal septicemia often follows. By combining the method of immunization with a subsequent alcoholizing and spraying of virulent organisms, infection and localization in the lung with lobar pneumonia may be produced. It must be remembered, however, that in this work at least three variables are always present: (1) the natural immunity of the individual mouse; (2) the artificial immunity produced by exposure to pneumococcus; (3) the effect of alcoholic intoxication upon the invasion of the pneumococcus, which differs from individual to individual. To produce the pneumonia a selected population was being employed. Furthermore, in these experiments death was the only token of infection recognized, no clinical data being employed, and it is probable that cases of infection occurred and were missed because the animals recovered. Despite all these variable factors, convincing results were obtained, results which furnish interesting epidemiological data.

The high incidence in the normal mice of serofibrinous pleurisy without demonstrable evidence of intrapulmonary localization is difficult to explain. It must be remembered, however, that lungs which show interstitial inflammation often appear only congested on gross examination, and consequently lesions may easily have been present in some one of the lobes which was not included in the portion studied microscopically.

The fact that mice immunized by exposure to a spray of dead organisms showed increased local resistance to subsequent infection by the inhalation method is all the more interesting since it has previously been found that mice immunized by this method present little general immunity to intraperitoneal inoculation. This may be due to a local active immunity in contrast to the instances in which immunization is effected with living organisms when the animals show a general immunity. Of even greater suggestive value is the observation that

mice which have been immunized by inhalation of one type of pneumococcus show evidence of increased immunity against organisms of a heterologous type when infection occurs by inhalation. This would seem to indicate that there is an antigenic fraction of the pneumococcus cell common to all types of pneumococci.

The fact that mice with a relatively high general immunity which have recovered from an intraperitoneal injection of 0.000,001 cc. of a virulent Type I culture may be infected by the inhalation of Type II pneumococcus would lead one to suspect that just as there may be an antigenic factor common to all types of pneumococci, so there may be a specific substance in the various types of pneumococci which plays an important rôle in determining infection. The ability to infect mice which are highly resistant to one type of pneumococcus with another type by inhalation may, in part, explain why in man one attack of pneumonia apparently confers no immunity against subsequent infections.

The results obtained support and extend Wadsworth's contention that to produce pneumonia there must be a subtle equilibrium between virulence of the infecting organism on the one hand, and susceptibility of the host on the other.

CONCLUSIONS.

1. Non-immune mice which are alcoholized and die of septicemia following exposure to a spray of virulent pneumococci rarely show any localization of infection in the lung.

2. Mice which have been partially immunized by previous inhalations of living or killed pneumococci and which while alcoholized are exposed to an atmosphere of virulent pneumococci often develop a pneumococcus lobar pneumonia.

3. Mice which have been rendered relatively immune to one type of pneumococcus may frequently develop lobar pneumonia after the inhalation of pneumococci of another type.

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PATHOLOGY OF EXPERIMENTAL PNEUMOCOCCUS PNEUMONIA IN MICE.

By ARNOLD BRANCH, M.D., and ERNEST G. STILLMAN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

Plates 29 to 31.

(Received for publication, July 28, 1924.)

In preceding papers (1) it has been shown that virulent pneumococci generally disappear from the lungs of normal mice within a few hours after inhalation and do not give rise to generalized infection. In alcohol-intoxicated mice, however, pneumococci persist in the lungs for a longer period and a fatal septicemia frequently follows. It has further been shown (2) that a high degree of immunity is produced in mice following repeated inhalations of live pneumococci and that a less marked immunity results from exposure to a spray of dead organisms. In the instances in which non-immune mice succumb as the result of infection following a pneumococcus spraying while alcoholized, death is due to general septicemia and there is no evidence of localized infection in the lung. However, mice which have been rendered partially immune by inhalation of live or killed pneumococci and are later alcoholized and exposed to virulent pneumococci show definite evidence of localized pulmonary infection.

In the present paper we shall report upon (1) the normal histology and the pathological lesions which have been encountered in routine examinations of the lungs of a large series of stock mice; (2) the effect of alcohol on the lungs of normal mice together with the changes brought about by the inhalation of pneumococci; and finally (3), the pathological lesions produced in the lungs of normal and immunized mice sprayed with pneumococci while under the influence of alcohol.

Technique.

At the time of autopsy sections of lung tissue were fixed in Zenker's fluid containing 5 per cent acetic acid. They were later embedded in paraffin and stained by Gram's and Mallory's eosin-methylene blue methods, respectively.

Normal Histology.—Except for occasional small or more extensive areas of congestion the lungs of normal mice appear salmon pink and crepitant. These congested areas have been noted by Branham, Maitland, Cowan, and Detweiler (4) and are ascribed to the methods whereby the animals were sacrificed. Certain points of histological difference from the human lung should be emphasized. In the mouse there are no secondary pulmonary septa and the pleura has no definite sub-pleural layer, but appears to consist of a single layer of endothelial cells covering the peripheral alveoli. Lymphoid tissue is sparsely distributed and lymphatics are not prominent, only becoming so when dilated, when they may be seen around blood vessels and bronchi.

Pathological Lesions in Stock Mice.—The lesion most frequently encountered was a chronic consolidation of the lung which occurred in about 3 per cent of apparently healthy stock mice. Both in the gross and microscopically the lesions were readily differentiated from the pneumococcus lesions to be described. These evidences of chronic pneumonic change are probably the result of some intercurrent infection, but no attempt has been made so far to isolate the etiological agent. The consolidated areas usually occur in multiple patchy fashion scattered through numerous lobes, and the right medial lobe is most often affected. They may, however, involve the whole of a single lobe. In the early stage the consolidated areas appear red, firm, dry, and liver-like. Later they appear grayish and gelatinous, almost transparent, and the surface is irregularly granular and puckered. Pleurisy occurred rarely.

On microscopic examination the most prominent and constant feature was a mononuclear reaction around the bronchi and blood vessels. These areas occurred around both large and small radicals as a mantle or collar and could be traced from the hilum outward. The accumulations of mononuclear cells probably represent a massive increase in the normally inconspicuous lymphoid elements, but large numbers of plasma cells were also present. Areas of true pneumonic consolidation also occurred, the exudate being composed predominantly of large mononuclear cells. As a rule, only a few polymorphonuclear leucocytes were seen in the alveolar exudate whereas the bronchial exudate contained many. The consolidated areas were often small and situated around the hilum, but when large extended from pleura to hilum, while occasionally the whole of a single lobe was involved. Fibrin was rarely found and the exudate was chiefly cellular. In some instances the exudate in the bronchi and alveoli was definitely necrotic, the focal changes bearing some resemblance to tuberculosis.

The Pulmonary Lesions Following Alcohol Administration.—In order to determine whether the administration of alcohol causes any demonstrable changes in the lungs, twelve animals, which had received alcohol either by spray or intraperitoneally, were killed at intervals and the lungs examined histologically. A few animals which had died of an overdose of alcohol were also examined. In no instance were any lesions found in the lungs of the alcoholized mice which did not occur in normal animals.

Histological Examination of Lungs of Mice Following Spraying with Pneumococci.—With a view of determining the relative number of organisms inspired by the inhalation method, twenty-five mice were killed at intervals of from 5 minutes to 24 hours after exposure to a pneumococcus spray. In the microscopic sections of the lungs of those mice killed shortly after exposure, large numbers of pneumococci were to be seen in the upper respiratory tract, especially in the pharynx, and also in the esophagus. They were found only occasionally, however, in the trachea and never in the bronchi or alveoli. That they were present, however, in the lower respiratory tract was proved by the fact that when small pieces of the periphery of the lobes were placed in broth, cultures of pneumococci were obtained. From this it is evident that the inspired bacteria reach the deeper parts of the lung following inhalation, but in such small numbers as to be demonstrable only by cultural methods.

Pathology of Pneumococcus Pulmonary Infections in Mice.—This report is based on the examination of the lungs of 133 animals which developed pulmonary lesions. The total number of animals exposed was over 900. In each instance in which death occurred following exposure to pneumococcus spray the organism was recovered from the heart's blood. The lesions may be conveniently discussed under (1) pleurisy and congestion, and (2) exudative pneumonitis.

1. Pleurisy and Congestion.—In 55 instances congestion and pleurisy were found in the absence of any exudative inflammation in the lobes of the lung examined. These lesions occurred about as frequently in the previously normal mice as in the animals immunized in various ways. Usually there was a considerable amount of serofibrinous fluid in the pleural cavities. One or both pleural cavities were involved while in some cases pericarditis was also present. Less often the exudate was fibrinous and formed a thin coating over the pleura of one or more lobes. The

lung itself was dark red and congested, and in the gross gave the impression of being consolidated. On microscopic examination the prominent feature was a widening and engorgement of the alveolar capillaries and an exudative pleurisy. Red blood cells and a granular serous fluid were sometimes found in the alveolar spaces. In no instance was any exudate of white cells encountered in the lung tissue proper, even in places contiguous to the pleura. Mononuclear cells were prominent in the earliest stage, whereas, later polymorphonuclear leucocytes predominated. The exudate varied greatly in its fibrin content, and pneumococci were found in large numbers especially in the superficial layer. The bacteria were chiefly extracellular, but occasionally within leucocytes. The most interesting feature was the sharp line of demarcation between the inflamed pleura and the congested lung. Thus, in sections which included the interlobar fissures, the inflammation was found to have spread along the pleural invaginations almost to the hilum of the lung, without any involvement of the adjacent lung tissue (Fig. 3). In a few instances the inflammation had also implicated the loose connective tissue around the hilum, peribronchial glands, and esophagus. We do not as yet understand the mode of primary invasion of the pleura by the pneumococcus. A study of serial sections of a whole lung is essential to this, and is being undertaken. In most instances no lymphatic involvement was evident in the lobe examined.

2. *Exudative Pneumonitis.*—An exudative inflammation was noted in the lungs of 78 mice. In eighteen pleurisy was also present. Twenty-seven of these instances were not considered in the paper preceding this one since they occurred in preliminary experiments in which accurate record of the method of immunization was not obtainable. The pulmonary lesions consisted of (1) interstitial inflammation, (2) red, and (3) gray hepatization, presumably the results of attempts on the part of the body to localize the infection.

In the pneumonias in which pleurisy occurred it was not infrequent to find the pleural exudate covering not only the surface of the consolidated lobe but also other lobes which merely showed congestion. In the early instances of interstitial inflammation the lungs often appeared merely congested or even normal on gross examination. In the stage of red hepatization one or more lobes were involved, sometimes on opposite sides. The lower lobes were most frequently affected. The affected lobes were moderately enlarged, dark red, firm and smooth (Fig. 1). On section they were moist and exuded serosanguineous material. In the gray stage the lobe was larger and of a dull grayish color (Fig. 2).

Microscopical examination showed that in the early interstitial inflammations congestion and edema were much more frequent when pleurisy was also present. The congestion was greater in the tissue surrounding the area of exudation than in the infiltrated area itself. Although the exudate in the interstitial tissue was rich in polymorphonuclear leucocytes, pneumococci could rarely be demonstrated histologically. The initial lesion was in the region of bronchioli, atria, and alveoli and these structures were involved even when the initial focus was near the hilum (Figs. 4 and 5). From this point in the lower respiratory tract the cellulitis

spread rapidly in irregular fashion either in the alveolar septa or in the interstitial tissue around the vessels and bronchi. These perivascular lesions were often extensive and were to be seen following the large blood vessels to their termination at the periphery of the lung. The perivascular lymphatics were greatly distended and filled with cell detritus, and there was often comparatively little actual interalveolar spread (Fig. 6); in other cases, however, the interalveolar spread was more marked than the perivascular, and the lymphatics were less involved. The lining of the bronchi was intact and their walls were rarely infiltrated. The lumina of the small bronchioli early contained an exudate rich in polymorphonuclear leucocytes. These cells apparently had emigrated from the initial interstitial foci. Vascular thrombi in capillaries and smaller vessels were occasionally seen but none were found in the larger vessels.

No sharp line can be drawn between the stage of interstitial inflammation and that of red hepatization. Frequently a typical area of red hepatization would be seen in one area while the adjoining showed a spreading interstitial inflammation. The stage of red hepatization was found as early as the 1st day but generally was encountered in mice dying on the 2nd day. At this stage the alveoli were filled with an exudate of red blood cells and polymorphonuclear leucocytes. Occasionally many mononuclear cells were noted. Fibrin was prominent in only a few instances. The alveolar walls now contained fewer inflammatory cells and the capillaries appeared engorged. In some instances fibrin could be seen in the capillaries. The numbers of pneumococci varied, sometimes only a few being present and in other instances large numbers.

As the transition from the stage of early interstitial inflammation to that of red hepatization was gradual, so was there a gradual transition from red to gray hepatization. In a single lobe areas resembling both red and gray hepatization frequently occurred. The stage of gray hepatization was reached at about the 3rd day. The alveoli were then completely filled with the polymorphonuclear and mononuclear exudate and the alveolar walls were anemic (Fig. 7). In some places the latter could be made out only with difficulty, the section showing what appeared to be a mass of clumped white blood cells. Vascular thrombi were not prominent although present in a few instances. Only occasionally were dilated lymphatics visible in the pleura. For these reasons observations on the lymphatics and the rôle they play were limited. The lymphatic system is apparently much less developed in the lungs of mice than of animals with secondary septa. The lymphatics, when distinguishable in the inflamed areas, were seen only in the perivascular and peribronchial tissue, and in some cases were filled with the cellular exudate.

Although the lesions described above are typical of the kind most frequently observed, other lesions were found which, in the hemorrhagic nature of the exudate, resembled more closely those described by Wadsworth (5) as occurring in the least immunized rabbits of his

series, and also by Hirsch and McKinney (6) in an epidemic of bronchopneumonia due to Pneumococcus Type I and Group IV. We are under the impressions that lesions of this type occurred, just as was the case with Wadsworth's rabbits, oftenest in mice with a relatively slight immunity as judged by the number of previous pneumococcus inhalations.

DISCUSSION.

There seem to be three factors necessary to produce pneumococcus pneumonia in mice, namely, the presence in the respiratory tract of a sufficient number of virulent organisms, penetration of the lung tissue, and some degree of local resistance or reactivity in the lung. This is, in some instances, apparently an expression of a general immunity rather than an allergic phenomenon. Mackenzie[1] has recently made the interesting observation that following reinjections of pneumococcus antigen intradermally, guinea pigs exhibit a marked local "hyperreactivity" (allergy) without developing a general immunity. As we were able to produce pneumonia in mice immunized by spraying dead organisms, which animals exhibit little general immunity to intraperitoneal inoculation, it may possibly be that the increased reactivity in the lung is in other instances an expression of a purely local immunity.

The initial lesion, when the reaction is focal, appears to be in the neighborhood of the alveolar ducts, atria, and alveoli. The infection spreads just as readily from the hilum outward as from the periphery inward. The earliest lesion is an interstitial inflammation or cellulitis which spreads in the interstitial tissues around the vessels and bronchi and in the alveolar walls. The lymphatics become filled with exudate but probably play a minor part in the spread of the infection, with the exception perhaps of the pleurisy. The extravasation of the exudate into the alveoli in the later stages of red and gray hepatization is a secondary phenomenon. The exact point of invasion of the organisms has not been determined beyond question, that is to say whether the pneumococci penetrate into the lower respiratory tract, in which case the infection would be a direct one, or lodge primarily in the upper respiratory tract. In the latter instance they would then reach the

[1] Mackenzie, G., personal communication.

lung by the lymphatics through reversal of the stream or pass through the lymphatic glands into the blood or enter the blood stream direct. In any of these events, lobar pneumonia would have to be considered, like acute splenitis, as a local reaction of an organ to a general septicemia.

Lobar pneumonia in mice is not of strict anatomical distribution, the infection spreading in the interstitial tissue like any other cellulitis until it is controlled. The area of demarcation is usually sharp, and although the disease tends to spread within the lobe in which it arises, it may readily extend into another lobe without involving the whole of the lobe first invaded. This is well known to be true in man also, in whom the margin of the most affected lobe may be perfectly healthy although part of another lobe, the middle lobe especially, also shows involvement by direct spread of the pneumonic consolidation.

It is interesting to note that the pleura often forms a sharp line delimiting the pulmonary lesion. One may see not infrequently a normal pleura overlying an inflamed lung. This state of affairs would seem to be indirect argument in favor of the pleural involvement being due to lymphatic rather than to direct spread of the infection.

MacCallum's (7) conception that the difference between lobular and interstitial pneumonias is largely a matter of degree of resistance in the tissue seems well founded. One can readily understand how it is that the pneumococcus is responsible for all of the lesions here described.

The comparative rarity in lobar pneumonia of true reinfection of the other lung, or parts of the same lung, may be explained by the assumption that even if the infectious agent reaches other parts of the lower respiratory tract, in the absence of some preceding injury, as for instance that produced by alcohol in the case of the experimental animals, invasion of the tissues fails to take place and the organisms are ultimately removed from the respiratory tract without causing harm. As an explanation of the "lobar" distribution of pneumococcus pneumonia the fact may be offered that penetration and infection only occur at some point where the normal defensive barriers have been broken down by a preliminary injury even when virulent organisms are present deep within the respiratory tract. This is a somewhat different conception from that offered by Blake and Cecil (8) or by

Permar (9). The authors first mentioned conceive of the organisms as entering the lung at the hilum and being carried along the lymphatics of a lobe to the alveoli where the interstitial lesion starts, while Permar interprets pneumonia as an exudative alveolitis, with the interstitial lesion as secondary and the spread mainly lymphatic. The lesions here produced in mice seem to be identical with those obtained by Blake and Cecil in monkeys, when one allows for the differences in anatomical structure of the two animals as further with those occurring in lobar pneumonia in man.

Alcohol may be taken as the prototype of many possible agencies which break down the effective protective barriers of the body and allow a penetration of organisms to occur. The exact nature of its action has not been determined. So far we have not succeeded in substituting other agencies.

The literature shows that previous attempts to produce pneumococcus pneumonia in mice have been unsuccessful because the necessary factors of penetration by the organism and partial immunity of the host were not present. In the small number of cases in which penetration was accomplished, the animals died of a general septicemia without localizing signs in the lung.

CONCLUSIONS.

1. The administration of alcohol produces no evident histological changes in the lungs of mice.

2. Following inhalations of pneumococci the organisms are not visible histologically in the lungs of mice either in the bronchi or alveoli.

3. In partially immunized mice which have been exposed to a pneumococcus spray while alcoholized, true lobar pneumonia not infrequently develops.

4. The primary lesion of such lobar pneumonia in mice is an interstitial inflammation of the alveolar wall and the infection spreads in the interstitial tissue like a cellulitis.

5. A tentative explanation of the "lobar" distribution of pneumococcus pneumonia is offered.

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EXPLANATION OF PLATES.

PLATE 29.

FIG. 1. Mouse A.B., 11–8. Red hepatization in the lung of an immune mouse. The animal had recovered from first treatment of alcohol intraperitoneally and inhalation of virulent Type I pneumococci. It died on the 3rd day following a repetition of the treatment. Natural size.

FIG. 2. Mouse A.B., 88–4. Gray hepatization in the lung of an immune mouse. The animal had been sprayed four times with virulent Type I pneumococci. After the usual interval it was given alcohol intraperitoneally and sprayed again with Type I pneumococci. It recovered. Again after the usual interval it was given alcohol intraperitoneally and sprayed with a virulent Type II culture It died 2 days later. Natural size.

FIG. 3. Mouse A.B., 57–3. Pleurisy and congestion without pneumonia in an immune mouse. The animal had been sprayed ten times with a virulent Type I culture. After the usual interval it was given alcohol intraperitoneally and sprayed with virulent Type I pneumococci. It died on the 2nd day. × 200.

PLATE 30.

FIG. 4. Mouse A.B., 2-4. Interstitial lung inflammation in a previously normal mouse. The animal was given alcohol intraperitoneally and sprayed with virulent Type I pneumococci. It died on the 2nd day. This illustrates one of the few cases in which pulmonary localization occurred in a previously normal mouse. × 315.

FIG. 5. Mouse A.B., 7-5. Early interstitial lesion around the atria in an immune mouse. It had recovered from a treatment of alcohol intraperitoneally and spray of viable atypical Type II pneumococci. After the usual interval it was given alcohol intraperitoneally and sprayed with virulent Type II pneumococci. It died 1 day later. × 450.

PLATE 31.

FIG. 6. Mouse A.B., 79-4. Interstitial inflammation showing perivascular lymphatics distended with degenerating polymophonuclears. Immune mouse sprayed six times with a culture of atypical Type II organisms. After the usual interval it was given alcohol intraperitoneally and sprayed with virulent Type II pneumococci. It died on the 2nd day. × 600.

FIG. 7. Mouse A.B., 99-18. Gray hepatization in an immune mouse. The animal had recovered from two previous treatments with alcohol intraperitoneally and inhalation of virulent Type I pneumococci. On a repetition of the treatment after the usual interval it died on the 2nd day. × 600.

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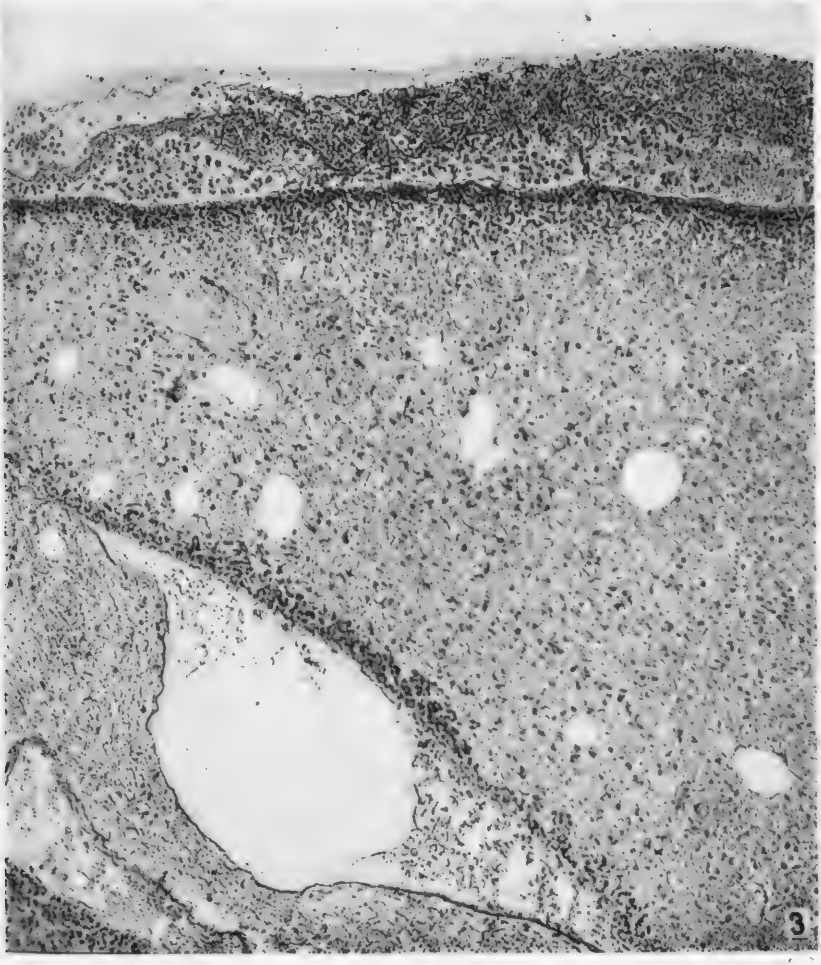

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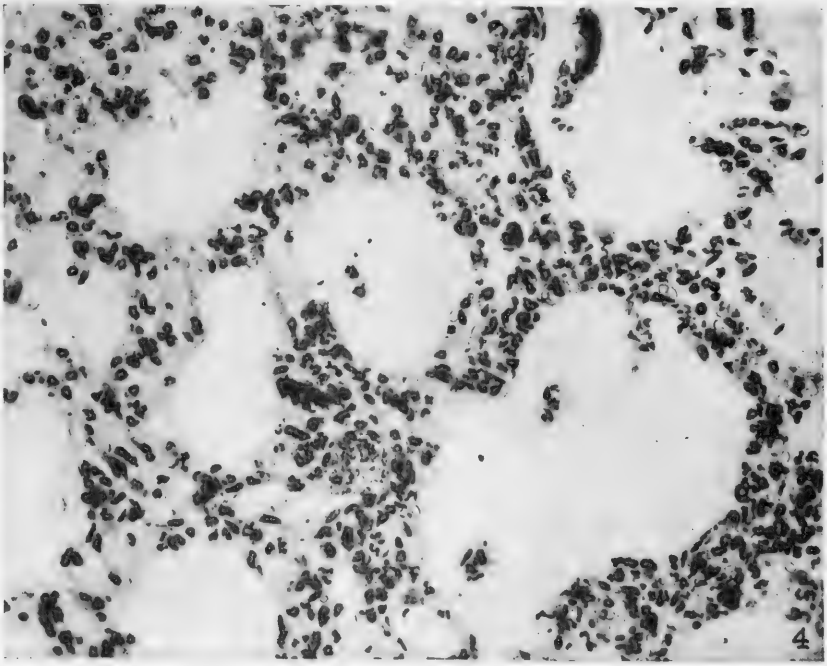
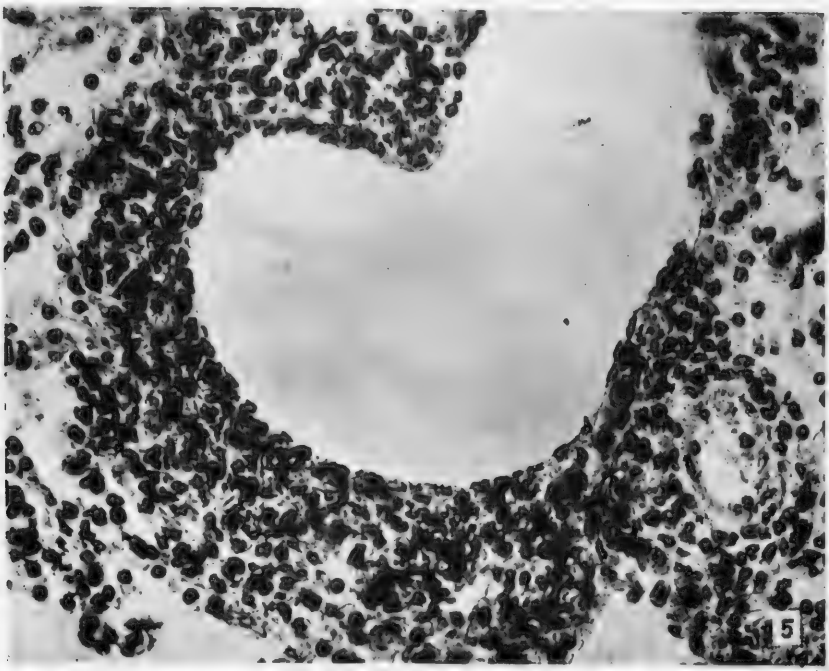
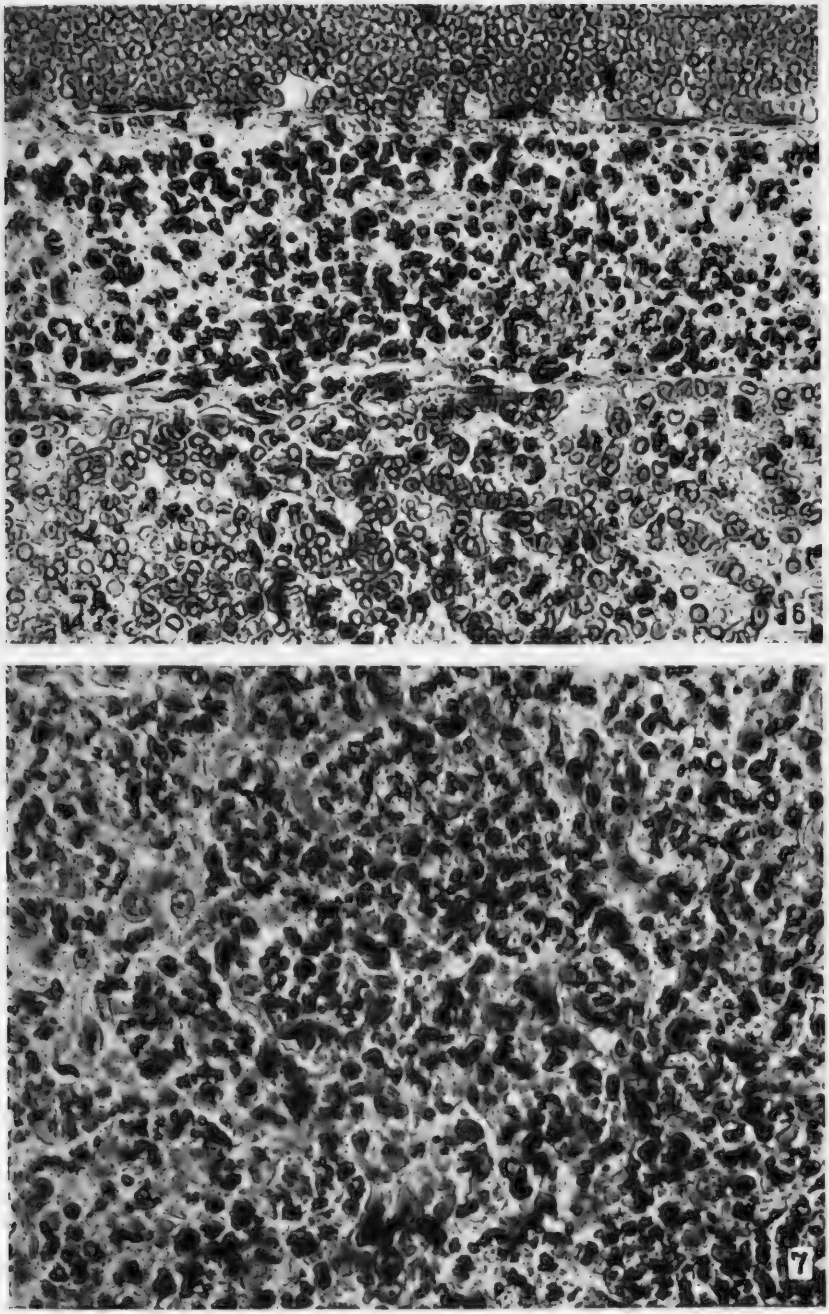
(Branch and Stillman: Pneumococcus pneumonia in mice.)





(Branch and Stillman: Pneumococcus pneumonia in mice.)



(Branch and Stillman: Pneumococcus pneumonia in mice.)

THE INFLUENCE OF THE SUPRARENAL GLAND ON THE THYMUS.

III. Stimulation of the Growth of the Thymus Gland Following Double Suprarenalectomy in Young Rats.

By HENRY L. JAFFE, M.D.

(*From the Laboratory Division of the Montefiore Hospital, New York.*)

Plate 32.

(Received for publication, July 23, 1924.)

It has been shown that double suprarenalectomy, in the rat and rabbit, if carried out after thymus involution had normally begun, brought about regeneration of the thymus gland (1–3). We pointed out that the regeneration probably began very soon after suprarenal ablation; that it was detectable early and progressed rapidly; and that it was completed in the rat in most cases within 2 weeks. These observations concerning thymus regeneration in older animals prompted us to study the effects of suprarenalectomy on the thymus glands of growing rats. We, therefore, suprarenalectomized animals the age of which varied between 35 and 54 days. The thymus of a normal rat increases progressively in weight and generally reaches its maximum size between the 80th and 90th days of life, and thereafter shows a gradual absolute decrease (4, 5).

We have been unable to find literature specifically concerned with experiments along these lines.

Methods.

All rats were reared in the laboratory from very tame albino stock (*Mus norvegicus albinus*), the original animals coming from the Wistar Institute. In our first paper we described the methods used in the care of our animals, the operative technique,[1] and the usual postoperative treatment. Autopsies were performed on all animals and a careful search made for suprarenal tissue. The thymus glands were removed as soon after death as possible and were dissected clean of the

[1] All operations were performed under ether anesthesia.

surrounding fat, connective tissue, and lymph nodes, and weighed. They were fixed in 10 per cent formalin, embedded in paraffin, and stained with hematoxylin and eosin.

We should like to emphasize again that our control and experimental rats were kept under identical laboratory and nutritional conditions at all times, and that standardization of these factors is extremely important in any research involving the thymus gland, as this gland is subject to considerable variation dependent upon these influences.

EXPERIMENTAL DATA.

The material includes a study of thirty-nine young rats, twenty-five of which were suprarenalectomized. Of these twenty-five, eleven died from 1 to 41 days after suprarenal extirpation, and were excluded from the experiments proper. The remaining fourteen animals were sacrificed at 2 week intervals from 2 to 8 weeks after operation, and the important data concerning them and their controls are summarized in the table. Of the fourteen control rats, eleven were litter mates of the operated animals.

The thymus gland of each of the fourteen suprarenalectomized rats, was heavier than that of its respective control, the difference varying from 12 to 85 per cent. The average thymic weight of the fourteen suprarenalectomized rats was 49 per cent greater than that of the control animals. On the other hand, if these weights are compared with Donaldson's standards of normal, we find that the average weight of the thymuses of the suprarenalectomized rats was 68 per cent above the normal, while that of the control rats was 12 per cent, making the difference between the average thymic weights of the suprarenalectomized and control animals + 56 per cent (6).

Concerning the eleven suprarenalectomized animals which died during the course of these experiments, and which were excluded from the calculations, three died within the 1st week after operation from acute postoperative effects; one died during the 2nd, and another during the 3rd week from suprarenal insufficiency; while six others died between the 4th and 6th weeks after operation. Although we pointed out previously that snuffles and progressive suprarenal insufficiency do not necessarily prevent regeneration of involuted thymus glands, we excluded these eleven rats because we wanted to avoid the complicating effects of these two conditions on the growing animals,

TABLE I.

Rat No.	Sex.	Length of time after suprarenalectomy.	Age at death.	Weight at death.	Major cause of death.	Weight of thymus.	Difference between thymuses of suprarenalectomized and control rats.
		days	*days*	*gm.*		*gm.*	*per cent*
R 39–3	M.	11	49	80	Back broken.	0.400	+81
R 42–1	"	Age control.	50	110	Sacrificed.	0.220	
R 46–2	F.	15	68	166	"	0.530	+80
R 48–2	"	Litter control.	68	150	"	0.293	
R 46–3	"	15	68	145	"	0.366	+26
R 48–4	"	Litter control.	68	120	"	0.290	
R 46–4	M.	15	68	185	"	0.635	+70
R 48–3	"	Litter control.	68	175	"	0.374	
R 37–4	F.	29	64	167	"	0.476	+54
R 40–1	"	Litter control.	64	123	"	0.308	
R 38–1	"	29	70	163	"	0.415	+60
R 40–2	M.	Litter control.	70	168	"	0.260	
R 39–1	"	29	70	163	"	0.369	+12
R 40–3	"	Litter control.	70	161	"	0.328	
R 38–3	F.	30	71	130	"	0.378	+55
R 41–2	M.	Age control.	70	155	"	0.243	
R 43–1	F.	31	77	143	"	0.418	+44
R 44–4	"	Litter control.	77	156	"	0.295	
R 44–3	"	31	74	145	"	0.479	+12
R 50–1	"	Litter control.	74	170	"	0.426	
R 47–1	"	41	86	160	"	0.312	+35
R 49–2	"	Litter control.	86	165	"	0.231	
R 44–2	"	55	98	210	"	0.552	+85
R 49–4	M.	Litter control.	98	255	"	0.298	
R 44–1	F.	55	98	150	"	0.375	+39
R 45–3	"	Litter control.	98	167	"	0.271	
R 43–2	"	55	101	152	"	0.392	+34
R 37–2	M.	Age control.	104	240	"	0.292	

since they would be reflected also on the growing thymus. It is noteworthy, however, that, in spite of the fact that some of the rats which died were extremely ill for 2 or 3 weeks, their thymuses were heavier than those of the Wistar rats of the same age.

Young rats withstand the late effects of suprarenal ablation much more poorly than do older animals. This is due to their greater susceptibility to intercurrent infections. Almost every surviving suprarenalectomized animal developed snuffles, and this undoubtedly contributed to the death of many. The number of deaths would probably have been much greater had we not sacrificed the animals at the scheduled time of 2, 4, 6, and 8 weeks after suprarenalectomy irrespective of their clinical condition. Among our control rats there was only one instance of severe snuffles, despite the circumstance that the control and operated animals were frequently kept in the same cages, and always under the same environmental and nutritional conditions.

The figure shows thymuses of twelve rats, six of which had been suprarenalectomized. Their thymuses are compared with those of their controls.

Histology.

Microscopical examination discloses no remarkable difference between the thymuses of the suprarenalectomized and control rats. The lobules are divided into prominent cortical and medullary zones, and the lobules of the thymuses of the suprarenalectomized rats are on the whole larger than those of the control animals.

DISCUSSION.

The results show that suprarenalectomy, if carried out in young rats 35 to 54 days of age, is an additional stimulus to the growth of the thymus gland. The average thymic weight of a series of fourteen suprarenalectomized rats is about 50 per cent above that of the average weight of fourteen controls. This thymic hyperplasia is apparent soon after suprarenalectomy; the maximum effect being attained usually within 2 weeks. To our knowledge the only other controllable experimental condition which brings about such marked prepubertal thymic hyperplasia is castration, but castration is not so

powerful a stimulus (2, 7–9). Here again, we are confronted with further evidence of the similarity in reaction of the thymus and lymphoid tissue both to suprarenalectomy and gonadectomy.

Prepubertal thymic hyperplasia following suprarenalectomy may offer a partial explanation of the pathogenesis of status lymphaticus. In the human cases hypoplasia of the suprarenals is one of the outstanding pathological findings together with a large thymus and prominent lymph nodes (10–12). While earlier authors emphasize the hypoplastic changes of the chromaffin tissue, the later work of Marine and Baumann on the rabbit and our work on the rat clearly indicate that the lymphoid overgrowth is dependent upon interrenal and gonadal insufficiency.

Of additional interest in this connection is the fact that an extraordinary degenerative change takes place in the suprarenal cortex of infants during the first month of life, these changes having been independently described by Thomas, Elliott and Armour, and Kern in 1911 (13–15). Their papers were soon followed by the more extensive reports of Landau (16), and Lewis and Pappenheimer (17). The anatomical descriptions of the above mentioned observers agree as regards the essential lesions. The degeneration is fully established at the end of the 2nd week of life and during the next 5 or 6 weeks rapid removal of the broken down cells takes place leaving the relatively thin glomerular layer intact. This layer collapses upon the medulla, and, according to Lewis and Pappenheimer, there is no appreciable increase in width of the cortex, that is, to say regeneration, up to the age of 3 years. The cause of this cortical destruction is not definitely known. Marine, Lowe, and Cipra (18) found that during the 2nd week of life in infants there is a rise in heat production, which they attributed to the involution of the suprarenal cortex on the basis of previous heat production studies on suprarenalectomized rabbits.

We are of the belief that the pathogenesis of the lymphoid state may be connected with excessive spontaneous cortical involution in infancy, or with a disturbance in the normal regeneration of the suprarenal gland following this spontaneous involution. It is conceivable that abnormal spontaneous involutions in infancy may lead to the production of hypoplastic suprarenals.

Hemicephaly is associated with high grade aplasia of the suprarenals and usually in addition presents a large thymus (14, 19–23). With regard to the suprarenal itself, most writers state that the aplasia concerns the medulla and cortex equally. Thus the suprarenal glands are abnormally small both in status lymphaticus and in hemicephaly, and in both enlargement of the thymus is present, which enlargement may stand, in regard to the suprarenals, in the relation of cause and effect.

CONCLUSIONS.

1. Double suprarenalectomy in growing rats is an additional stimulus for thymic growth and brings about marked prepubertal hyperplasia of this gland.

2. Our results on young rats suggest that the suprarenal cortex may be a factor in the excessive thymic hyperplasias seen in infants and children.

I wish to express my appreciation for the helpful and suggestive criticism of Dr. David Marine.

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EXPLANATION OF PLATE 32.

FIG. 1. Showing thymuses of young suprarenalectomized rats compared with their controls.

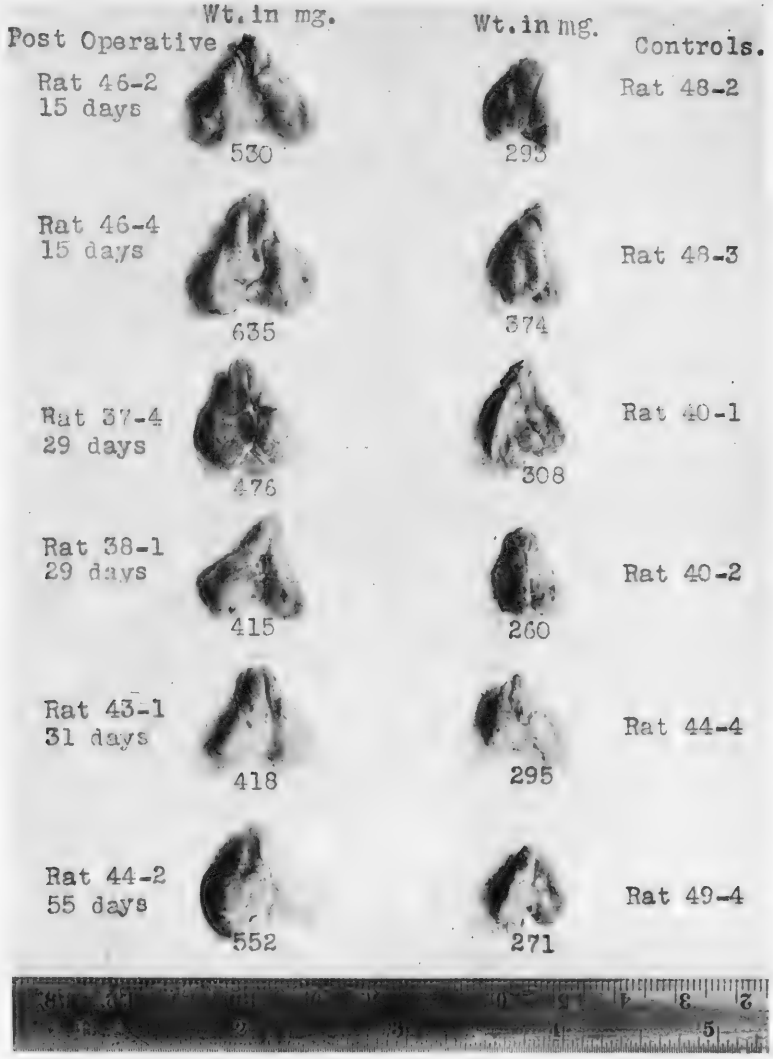

FIG. 1.

(Jaffe: Influence of suprarenal gland on thymus. III.)

THE PRODUCTION OF AN EXOTOXIN BY CERTAIN STRAINS OF STAPHYLOCOCCUS AUREUS.

By JULIA T. PARKER.

(From the Departments of Bacteriology and Pathology of the College of Physicians and Surgeons, Columbia University, New York.)

PLATE 33.

(Received for publication, July 15, 1924.)

Following Dick and Dick's (1) recent discovery that scarlet fever strains of *Streptococcus hæmolyticus* produce a dermotoxic poison for man, the possibility of demonstrating that strains of *Staphylococcus aureus* produce a similar poison presented itself. It is not necessary to review here the literature on the various poisons found in filtrates from broth cultures of *Staphylococcus aureus*. The hemolysins (which can be demonstrated in broth cultures of *Staphylococcus aureus*) probably have no bearing in the present relation, as many of the non-poison-producing strains, ineffective in rabbits, were markedly hemolytic. On the other hand, the leucocidins appear to resemble the above mentioned dermotoxic poison in many respects (2–4), such as thermolability and neutralization by immune and non-neutralization by normal rabbit serum.

After many unsuccessful attempts, we succeeded in demonstrating in sterile filtrates of broth cultures of certain strains of *Staphylococcus aureus* a powerful poison, with a selective action for the skin. The poisonous filtrates have not been examined for destructive action on leucocytes, and therefore there is no evidence as tc whether they do or do not contain leucocidins. The following report gives our results to date with the action of this poison on the skin of rabbits. Its effect on the human skin has not as yet been worked out.

Sources of Cultures.—Although twenty-one strains of *Staphylococcus aureus*, isolated from various conditions, were tested, from only four of these were filtrates obtained, which were effective on the skin of rabbits. We have no evidence as to whether, by varying experi-

mental conditions or by concentration of filtrates of the other seventeen strains, similar skin-poisoning products could be demonstrated. This statement applies only to the effects on rabbits, as one of these rabbit-negative strains produced an effect in human skin similar to the Dick reaction.

TABLE I.

Staphylococcus aureus Strains Studied for Poison Production.

No.	Date of isolation.	Condition isolated from.	Amount of skin poison demonstrated in broth filtrates.
1	Oct., 1923	Boil.	0
2	1924	Dermatitis.	0
3	1924	Carbuncle.	0
4	Apr., 1924	Dermatitis.	0
5	1924	"	0
6	1924	Boil.	++++
7	1924	Dermatitis.	+++
8	1924	Abscess.	0
9	1924	Dermatitis.	++
10	1924	Abscess.	0
11	1924	"	0
12	1924	Dermatitis.	0
13	1924	"	0
14	1924	"	0
15	Apr., 1924	"	0
16	1924	Boil.	0
17	1924	Septicemia.	0
18	1924	Dermatitis.	0
19	1924	"	0
20	Nov., 1922	Carbuncle.	+
21	1924	Dermatitis.	0

In the above table + signifies slight yield of poison; ++ signifies moderate yield; +++, large amount; ++++, very large amount.

The sources of the *Staphylococcus aureus* strains studied and the dates of isolation are given in Table I.

Preparation of Media.—The poison is produced by the growth of *Staphylococcus aureus* strains, presented in Table I, on any well buffered broth medium containing only a small amount of glucose.

It was found that the addition of 1 per cent glucose prevented poison production and that in media containing traces of glucose toxicity appeared late—probably

after the sugar was destroyed. This may be explained by the protein-sparing action of glucose demonstrated by Kendall (5), or by the fact that it was impossible to buffer sufficiently to prevent much acid formation and resulting inhibition of growth. Poison production was also dependent on the maintenance of a hydrogen ion concentration between 6.8 and 8.2. In broths allowed to become more acid or alkaline no substance toxic for rabbit skin was found. The medium, from which our most powerful poisons were obtained, was prepared by adding an equal volume of M/15 phosphate buffer solution, pH 7.4, to ordinary sugar-free broth containing 4 per cent Witte peptone. The resulting buffered broth was brought to a boil, filtered through paper to clear, distributed in Ehrlenmeyer flasks, and autoclaved for 15 minutes at 15 pounds pressure.

After inoculation with a suitable strain of staphylococcus, the poison can be demonstrated in the sterile Berkefeld filtrates of this broth after 24 hours growth, but the most toxic poisons were regularly obtained after 4 to 6 days growth. If growth is allowed to continue longer than 6 days, the filtrates become progressively less poisonous. As there is no change in H ion concentration even after 8 days growth on this medium, the deterioration of the poison cannot be related to any change in pH. The lessened toxicity of the older filtrates may be due to several factors,—diminished growth with resulting smaller amount of poison produced, deterioration of formed poison by prolonged incubation at 37°C., and destruction of poison by autolytic enzymes set free from disintegrating organisms. We have found that whenever there is autolysis in broth culture as evidenced by finding Gram-negative organisms in smears, the filtrate is comparatively atoxic.

Inoculation of Animals.—The toxicity of the filtrates was tested by intracutaneous inoculation in rabbits.

When white, gray, or black rabbits with thin white skins were used, there was practically no variation in the reaction which any one poison produced. Throughout this work, 0.1 cc. of the filtrate to be tested was inoculated intradermally on the back or side of the animal. At the same time a control of 0.1 cc. of uninoculated broth was always injected into the same rabbit. Six to fourteen tests can be made in one animal.

Characteristic Appearance of the Reaction.—The reaction produced by a toxic filtrate is striking and follows a definite course. As compared with control broth or non-toxic filtrate, a toxic filtrate diffuses rapidly, the wheal disappearing immediately after the inoculation. In many rabbits there is a definite blanching zone 1 to 2 hours after the injection of poisonous filtrate, which spreads beyond the original wheal raised by the injection. 3 to 4 hours after the inoculation there is in all rabbits, both in those that do and those that do not

give the blanching reaction, a dark bluish purple circumscribed area of 2 to 5 cm. in diameter, depending on the toxicity of the filtrate. The next day the purple color assumes a yellow tinge and there is added a wide deep red zone of 0.5 to 3 cm. surrounding the yellowish area. At this time, with strong poisons, there is much edema spreading down towards the abdomen. By the 3rd or 4th day, the yellowish area of 3 to 5 cm. has become progressively yellower, as if necrotic, and is slightly raised as compared with the surrounding border of red. By about the 5th day, brown patches appear in the yellow area and increase in size, until, at about the 20th day, the whole lesion has become a dark brown dry scab with an encircling rim of red. 4 to 8 weeks later, the scab falls off, leaving an ulcer. If a large amount (2 to 3 cc.) of poison is injected intradermally and immediately subcutaneously, the reaction is similar to the one just described, except that the area covered is proportionally larger.

Control broth or filtrates of non-poison-producing cultures give no reaction of any kind, except occasionally a slight redness on the area of inoculation, which disappears in 24 to 48 hours. Large amounts of control broth or non-toxic filtrates (3 to 5 cc.) injected intradermally and immediately subcutaneously may produce a slight redness on the area of inoculation, but this redness could not be mistaken for even a very slight reaction caused by the injection of a poisonous filtrate.

Whether or not a skin reaction is obtained when a poison is injected subcutaneously, under the superficial fascia, seems to depend entirely on the strength of the poison. The subcutaneous inoculation of even a large amount (2 to 3 cc.) of a weak poison produces no reaction or only a slight redness over the area of inoculation, whereas the subcutaneous injection of 0.1 cc. of a potent poison produces a reaction which is only slightly less than that obtained from the intradermal inoculation of the same amount of powerful poison.

On the other hand, the reaction caused by any one poison is the same, whether it is injected intradermally or immediately subcutaneously; that is to say, between skin and superficial fascia. This is an important point because it makes possible the injection of large amounts of poison and the obtaining of reactions corresponding to the quantity and strength of poison inoculated.

Histological Changes in the Skin Reactions.—I am indebted to Dr. William C. Johnson for the histological work.

While no attempt has been made to carry out a complete or detailed study of the tissue changes in the skin at the site of injection of the toxic filtrate, sections have been made from a few rabbits, showing various stages of the reaction.

In the section of a lesion taken ½ hour after injection no pathological alteration is discernible except slight edema, which extends through the whole corium, and a scattering infiltration with a very few polymorphonuclear leucocytes and large mononuclear cells. Section of a lesion 24 hours old shows marked evidence of inflammation and beginning necrosis. Through a circumscribed area over the site of injection the epidermis is thin, the nuclei of the epithelial cells are shrunken and pycnotic, and the staining reaction of the cytoplasm of the cells has become more acidophilic. The corium, especially just beneath the epidermis, shows a marked infiltration with polymorphonuclear leucocytes, and a few mononuclear cells. Scattered through the corium are many foci where the connective tissue fibers appear to be fragmented, and infiltrated with leucocytes. The cellular exudate shows a marked amount of pycnosis and nuclear fragmentation. In the superficial layer of the corium the capillaries are slightly congested. In the deepest part are several small arteries and veins whose walls show necrosis and infiltration with leucocytes. A few of the vessels are thrombosed. In the layer of cutaneous muscle beneath the corium many fibers show necrosis, fragmentation, and invasion by leucocytes.

Later stages of the lesions, as seen in sections 3, 4, 5, and 21 days after intradermal injection of the toxin, show more marked infiltration with leucocytes, and complete necrosis of a mass of tissue including a sharply circumscribed area of epidermis, together with the underlying corium, extending about half way down to the cutaneous muscle layer. As the necrotic tissue dries up, forming a scab, it becomes very sharply demarcated from the underlying living tissue by the accumulation within it of a broad dense zone of disintegrating leucocytes.

In the remaining portion of corium beneath the necrotic mass, reparative activity is shown by a proliferation of fibroblasts. The walls of some of the small arteries and veins in this layer are partly necrotic, and are infiltrated with polymorphonuclear leucocytes and mononuclear cells.

Evidence of Systemic Poisoning.—Only irregular results were obtained by the intravenous injection of the poison into rabbits. Usually there was no effect even when comparatively large doses (3 to 8 cc.) of a powerful skin poison were given to rabbits of over 1,300 gm. On the other hand, two very young rabbits, weighing 1,030 and 710 gm. respectively, died less than 2 hours after the intravenous

inoculation of a strong poison. 2 cc. of control broth were innocuous to two rabbits, each weighing 630 gm.

The poison appears to be definitely toxic when given intradermally. Usually the animal seems sick and drowsy, and will not eat. This condition begins ½ to 2 hours after injection and lasts for several days. About 25 per cent of the rabbits used, when a relatively large amount of poison was injected, died 3 to 5 days later. At autopsy nothing could be seen macroscopically to account for these deaths except extreme emaciation.

Microscopically, in one of these rabbits dying on the 2nd day after injection, the heart muscle showed numerous areas of focal necrosis. A few focal necroses were present in the liver. An interstitial nephritis present in this rabbit was thought to be probably independent of the experiment. No other visceral lesions were noted.

Properties of the Poison.—The poison is extremely labile, being partly destroyed if heated to 50° for 1 hour and completely destroyed if heated to 55° for 1 hour. The poison may be preserved with only slight deterioration for at least 2 months, if kept in the dark at 4°C.

Antiserum to the Poison.—A question of importance to determine was whether a neutralizing antibody could be produced for our poison, and, furthermore, whether such neutralization, if it occurred, would take place in multiple quantities. Although to date we have immunized no animals systematically with the poison, we find that the serums of many of the rabbits used previously in this work in testing out filtrates for skin-injuring substances easily neutralize in small amounts our most powerful poisons. Normal rabbit serum, even undiluted, incubated with equal amounts of even a weak poison has no detoxicating effect.

That an antiserum produced against our poison neutralizes it in multiple quantities is evident from the following experiment.

Experiment 1.—Preliminary titration demonstrated that 0.02 cc. of a certain toxic filtrate produced a marked reaction (4 by 4 cm.) when inoculated intradermally into a normal rabbit. Following this, it was found that 0.002 cc. of Immune Rabbit Serum 18 when incubated with 0.02 cc. of the poison completely neutralized it, while 0.001 cc. of Serum 18 had only slight neutralization effect. Table II gives the results obtained by incubating 0.002 cc. of Serum 18 with 0.02 cc. of the poison and multiples of both the reagents, and inoculating these mixtures along with controls into a rabbit. The volume injected in each case was 0.2 cc.

Normal rabbit serum has no neutralizing effect on the poison except when incubated with it in comparatively large amounts. This is shown in Table III in which the same poison was used as in Table II.

From the experiments recorded in Tables II and III, we believe that there is only one interpretation of our results; namely, that our poison is a true toxin because its injection into rabbits stimulates the production of antitoxin.

TABLE II.

No.	Serum 18.	Poison.	Result. Reaction of rabbit.
	cc.	*cc.*	
1	0.001	0.02	++
2	0.002	0.02	0
3	0.01	0.1	0
4	0.5	5	0
5		0.02	++++
6		0.1	+++++

In Tables II and III, 0 signifies no reaction in rabbit; ±, red reaction of 2 by 2 cm. lasting at least 3 days; ++, reaction of 3 by 3 cm.; +++, reaction of 3.5 by 3.5 cm.; ++++, reaction of 4 by 4 cm.; +++++, reaction of 4 by 6 cm.

TABLE III.

No.	Normal rabbit serum.	Poison.	Result. Reaction of rabbit.
	cc.	*cc.*	
1	0.1	0.005	+++
2	0.5	0.005	±
3	2	0.005	0
4		0.005	+++
5		0.02	++++

The Toxin Is Identical from the Three Poison-Producing Strains Studied.—There is considerable variation in the potency of the filtrates produced by the four different strains (see Table I), but no variation in the character of the reaction, which is strikingly alike in all toxic filtrates.

By using the serum of a rabbit which had been inoculated with toxic filtrates from our Strain 7 only, and testing it against the poisons produced by two other strains (Nos. 6 and 20), we have found that it

neutralizes all three toxins equally well; and we therefore conclude that the toxins are identical in the strains tested. The fourth strain (No. 9) has not as yet been tested against our Serum 7.

Immunization of rabbits.				Skin reactions
1	2	3	4	5
Rabbit No.	Approximate total No. of skin units injected.	Method of injection.	Dates of immunizing injections.	Dates of obtaining serums and doing skin tests on rabbits.
1				May 26
2				" 26
3				" 31
4				" 31
5				" 31
6				" 31
7				" 31
8	5	Ic.	May 9	" 26
9	5	"	" 5	" 26
10	5	"	" 5	" 26
11	10	"	" 1	" 26
12	10	"	" 1	" 26
13	5	"	Apr. 25, 30	" 26
14	120	"	Mar. 17, Apr. 25, 30, May 13	" 26
15	80	50 ic., 25 iv.	Apr. 17, 25, May 13	" 26
16	75	5 " 20 subc., 50 iv.	" 15, 25, 30, May 2	" 26
17	110	60 ic., 50 ip.	" 15, 25, May 13	" 26
18	80	Ic.	" 15, 25, 28, May 13	" 26
19	155	"	" 15, 17, 22, " 13	" 26
20	155	"	" 15, 17, 25, 28, 30, May 13	" 26
21	200	"	May 23	" 31
22	150	"	" 20	" 31
23	60	"	" 15	" 31
24	550	"	" 21	" 31
25	1,500	"	" 14	" 31

0 indicates no reaction; ±, red reaction 2 by 2 cm. lasting at least 3 days; +, erythema; +++, yellow center 3 by 3 cm. to 4 by 4 cm.; ++++, yellow cente

Ic. indicates intracutaneous inoculation; iv., intravenous inoculation; ip., intraperi

* Rabbit 18 gave a 0 reaction to 50 skin units injected intradermally on May 13 a

Effect on the Skin Reaction of Circulating Antitoxin.—That the skin reactivity to the toxin of any treated rabbit is dependent on the amount of antitoxin which its serum contains appears probable from

Titration of serums for neutralization capacity.

8	9	10	11	12	13	14
Reaction of normal rabbit with 0.02 cc. of serum + 50 skin units.	Reaction of normal rabbit with 0.04 cc. of serum + 50 skin units.	Reaction of normal rabbit with 0.1 cc. of serum + 50 skin units.	Reaction of normal rabbit with 0.1 cc. of serum + 10 skin units.	Reaction of normal rabbit with 0.1 cc. of serum + 2.5 skin units.	Reaction of normal rabbit with 0.1 cc. of serum + 1 skin unit.	Approximate No. of antitoxin units in 1 cc. of serum.
++++		++++	+++	++	+	0
++++		++++	+++	++	+	0
				++	+	0
				++	+	0
				+	±	?
				++	+	0
				++	+	0
++++			±	0		100
			±	0		100
			+++	0		40
			0	0		100
			++	0		40
			++	0		40
±						2,500
++	0					1,250
++++		++++	++	+	±	?
++	0					1,250
						5,000
++						1,250
±						2,500
			+++	+	0	10
			+++	+	0	10
			+++	+		?
		++	±			100
		±				500

2 by 2 cm. surrounded by erythema; ++, yellow center 3 by 3 cm. surrounded by o 6 by 6 cm.

tion; subc., subcutaneous inoculation.

death on May 21.

Table IV, in which we have correlated the results of skin tests on previously treated rabbits with the amount of antitoxin which the serums of these same rabbits contain.

The rabbits were taken at random from those which had been used previously for the determination of toxicity of the filtrates. They were bled from the ear and inoculated intracutaneously in a fresh area with 0.1 cc. of a powerful toxin on the same day. The results of the skin tests on these rabbits are given in Table IV, Column 6. The titration of their serums for antitoxin is shown in Columns 7 to 14; the figures in the last column show the approximate number of antitoxin units contained.

The same toxin was used throughout the experiment, both for retesting the treated rabbits (Column 6) and for determining the antitoxin content of their serums. The potency of the toxin was such that 0.1 cc. of 1:50 dilution (0.002 cc.) produced a slight but typical reaction (yellow area 2 by 2 cm. surrounded by erythema) when inoculated into a normal rabbit. In the table we arbitrarily designate this amount of toxin as one unit, and the reaction obtained with it as a + reaction.

The titration of the antitoxin was carried out as follows: 0.1 cc. of varying dilutions of serums was mixed with 0.1 cc. of varying dilutions of toxin and incubated in the water bath at 37°C. for 1 hour. Simultaneously control mixtures were made with toxin alone diluted with salt solution and also toxin plus normal rabbit serum incubated at the same time. After incubation, normal rabbits were injected intradermally with 0.2 cc. of the mixtures and of the control toxin. One antitoxin unit was taken to be that amount of serum which would neutralize one unit of toxin.

The reactivity of every normal rabbit was controlled with at least one intracutaneous inoculation of the toxin dilution alone.

In Table IV, in Columns 2, 3, 4, and 5 are given the approximate number of skin units with which the rabbits had previously been injected, the method of injection, the dates the immunizing doses were given, and the dates of obtaining the serum and carrying out the skin tests recorded in Column 6. In Columns 7 to 13 inclusive are given the results of the titration of antitoxin. In Column 14 is given the approximate number of antitoxin units in 1 cc. of serum as calculated very roughly from the results of Columns 7 to 13. The antitoxic content of the serums was estimated by taking the amount of serum which neutralized a definite number of toxin units (produced ± or 0 reaction when inoculated together in a normal rabbit after incubation) and calculating from this the approximate number of toxin units which 1 cc. of the serum would neutralize.

The points worthy of note in Table IV are the following.

(*a*) The skin reactivity of any one rabbit to the toxin was found to be inversely proportional to the amount of antitoxin its serum con-

tained. There was one exception, Rabbit 8 which gave only a + reaction although its serum contained little antitoxin. Two possible explanations may be offered. This animal was much emaciated and its weak condition may have lessened its capacity to react to the poison. It was also an unfavorable subject for tests on account of its reddish brown color. It had also reacted unsatisfactorily to the immunizing injection (May 9).

(*b*) Under the conditions of this experiment, normal rabbit serums do not neutralize the toxin.

TABLE V.

No.	Approximate total No. of skin units injected.	Method of injection.	Dates of immunization.	Dates of obtaining serums.	Skin reaction with 25 skin units on June 24.
1	375	Iv.	May 29, June 2, 5, 12	June 21	++++
2	435	"	June 2, 5, 9, 12	" 21	++
3	375	"	May 29, June 2, 5, 12	" 21	++++

Titration of Serums for Neutralization Capacity.

No.	Reaction with 0.05 cc. of serum plus 25 skin units.	Reaction with 0.1 cc. of serum plus 25 skin units.	Reaction with 0.1 cc. of serum plus 5 skin units.	Reaction with 0.1 cc. of serum plus 1 skin unit.	Antitoxin units in 1 cc.
1	++ ++	++++	+++	+	0
2	++++	++++	±	0	50
3	++++	++++	+++	+	0

(*c*) Although there were two instances (Rabbits 24 and 25) in which a definite amount of antitoxin was produced after previous intracutaneous injections of toxin carried out all on one day, the most potent serums were those obtained after a series of inoculations spread out over a period of time.

Immunization by the Intravenous Route Elicits the Production of Very Little, If Any, Antitoxin.—From Table IV, it is evident that the stimulation of antitoxin is easily brought about by three or more intradermal injections of toxin. The next question, one of both practical and theoretical importance, was whether the intravenous injection of toxin would also elicit the production of antitoxin. Table V records

the results obtained in investigating this point. For details of this experiment the reader is referred to the explanation of the previous experiment, Table IV.

Although only three rabbits were immunized by the intravenous route, the results appear convincing as far as they go. Antitoxin could only be demonstrated in the serum of one of the three rabbits immunized, and in this serum the amount of antitoxin present was very small. This experiment (Table V) indicates that, for the production of antitoxin, the intradermal method of injection is far superior to the intravenous.

CONCLUSIONS.

1. The sterile Berkefeld filtrates of broth cultures of certain strains of *Staphylococcus aureus* have a selective poisonous action for the skin of rabbits.

2. The poison is thermolabile, being completely destroyed when heated to 55°C. for 1 hour.

3. The poison when injected intradermally into rabbits stimulates the production of antitoxin. We therefore conclude that the poison is a soluble toxin.

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EXPLANATION OF PLATE 33.

FIG. 1. Reaction in a normal rabbit 2 days after the intradermal injection of 0.1 cc. (25 toxin units) of *Staphylococcus aureus* toxin. ×½.

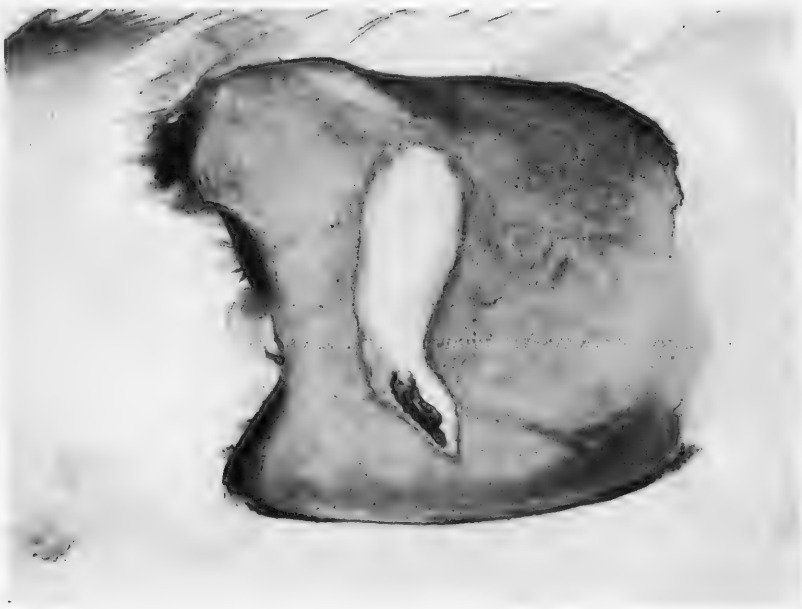

FIG. 1.

(Parker: Exotoxin produced by *Staphylococcus aureus*.)

A FILTERABLE VIRUS INFECTION OF RABBITS.

I. Its Occurrence in Animals Inoculated with Rheumatic Fever Material.

By C. PHILIP MILLER, Jr., M.D., C. H. ANDREWES, M.D., and HOMER F. SWIFT, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

Plates 34 and 35.

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INTRODUCTION.

Recently one of us (1) has reported upon a series of unsuccessful attempts to reproduce experimental rheumatic fever in rabbits and guinea pigs by intravenous, intraperitoneal, or subcutaneous injections of various materials from patients suffering with this disease. Subsequently different methods (2) were employed which in the hands of other workers have been successful in adapting to growth in laboratory animals certain viruses and microorganisms highly parasitic for the human host. The anterior chambers of rabbits' eyes and rabbits' testicles were injected with blood and joint fluid obtained from patients in the early acute stages of rheumatic fever and with verrucæ of acute rheumatic endocarditis recovered post mortem. The intraocular inoculations proved to be unsatisfactory because the inflammatory reaction could not be differentiated from that incited by the introduction of non-rheumatic material; but in certain of the testicles thus inoculated a diffuse subacute inflammation occurred which was transmissible from animal to animal by inoculation of the bacteria-free testicular emulsions. Control experiments, however, demonstrated the appearance of a similar orchitis in the fourth and fifth transfers of two series; in one the first animal was injected with normal human blood and in the other with the heart valve of a healthy dog. This orchitis could be carried on by the same method of transfer at intervals of about 1 month. With the exception of occasional

contaminants, cultures of the testicular emulsions were sterile on ordinary media. Dark-field examinations revealed no bacteria or spirochetes. No cell inclusion bodies were ever found in the lesions.

In 1923, as outlined in an earlier paper (3), the present authors continued the search for a virus in the blood and joint fluid of patients with rheumatic fever. As in the previous experiments, rabbits' testicles were inoculated, but the technique was modified by shortening the time between transfers, and by x-raying the rabbits or treating them with benzene. The latter procedure was carried out on the assumption that injury to the hematopoietic system might lower the resistance of the animals to experimental infection. Winternitz and Hirschfelder (4) found that pneumococcus infection was very acute and rapidly fatal in rabbits the bone marrow of which had been rendered aplastic by benzene poisoning. Hektoen (5) demonstrated that repeated doses of benzene greatly reduced the antibody production (lysin and precipitin) in rabbits and rats.

Methods.

The benzene treatment consisted of four to six daily subcutaneous injections of a mixture of equal parts of benzene and olive oil; for the first two doses the animals received 1.5 or 2 cc. of benzene per kilo body weight and subsequently 1 cc. per kilo. This period of benzene treatment was sufficient to lower the leucocyte count in most rabbits to less than 2,000 per c.mm. In the one x-rayed series the animals were exposed daily for 7 days and then every other day until inoculation within another week. In succeeding series of experiments, the intervals between transfers were progressively shortened (see Table I); 4 day intervals were found to yield the best results.

The substances injected were obtained from patients with rheumatic fever during the acute stages of the disease; they included blood, joint fluid, and unfiltered throat washings. Cultures of the blood and joint fluids showed no growth in ordinary media; the throat washings were not cultured. The rabbits were healthy adult males weighing usually from 1.5 to 2.5 kilos. 1 to 2 cc. of the material was injected into one or both testes of two or three rabbits. Thereafter the testicles were examined daily; and daily rectal temperatures taken. The rabbits were killed by a blow on the occiput; the abdomen was opened and the testicles removed aseptically by that route; when kept alive for immunity tests the animals were etherized and castrated with aseptic precautions. Upon removal from the body the testicles were placed in sterile dishes, inspected, and sectioned. A piece of each was fixed in Zenker for subsequent microscopic examination and two pieces were reserved for culture; the balance was used for transfer inoculation. When the

testicles of only one rabbit showed macroscopic changes suggestive of infection they alone were used for transfer. Otherwise the testicles of the two or three rabbits were combined for purposes of transfer. The organs were minced to a fine pulp with scissors, ground in a mortar with sand and 5 cc. of Locke's solution, and centrifuged a few minutes at low speed to throw down the sand. The resulting supernatant suspension was then used for transfer inoculation in amounts of 1 to 1.5 cc.

Aerobic and anaerobic cultures were made of each testicle in the following manner: one piece was cultured in an ordinary dextrose broth tube (5 cc.), the other in a deep Noguchi tube of dextrose broth (10 cc.) sealed with vaseline. It had been found that methylene blue added to these sealed tubes was decolorized by the following morning. Except for very occasional contaminations the cultures showed no growth. Several dark-field examinations of the testicular suspension were negative for spirochetes.

RESULTS.

The results are summarized in Table I. In four of the twelve series there arose in either the 4th, 5th, 6th, or 7th generation[1] certain morbid changes which, once they had appeared, recurred in subsequent generations. These changes were attributed to an infection, the cause of which was not demonstrable but will be referred to as a virus; its exact nature will be discussed later. Of the six series in which transfers were made every 4 days, three were positive. Of the six series in which transfers were made at intervals longer than 6 days, all but one were negative and this was also considered negative at the time; but reexamination of the histological material with the more exact standards of diagnosis later available (*i.e.* finding of nuclear inclusion bodies) made it probable that this series also was really positive.

Clinical Manifestations of Infection.—As a rule, the first manifestation of infection was discovered only after histological study of the testicles. Almost invariably, however, clinical evidence of infection appeared within the 2 succeeding generations. While transient swelling of the testicle frequently followed injection of any material, infected rabbits showed, in addition, increasing fullness and firmness of the testicles appearing about the 3rd day, oc-

[1] The rabbits originally inoculated are called the 1st generation; the first transfer inoculation, the 2nd generation, etc.

casionally accompanied by edema of the scrotum. Fever occurred in about half the infected rabbits. The temperature of normal rabbits varies so much that it is difficult to establish a criterion of fever. Of 82 rabbits which were certainly infected, 47 per cent showed a rectal temperature of over 103.5°F., while 23 per cent had fever of over 104°; in comparatively few (10 per cent) the temperature rose to 105° or more. Often undoubtedly infected animals showed neither local macroscopic nor general manifestations of disease.

TABLE I.

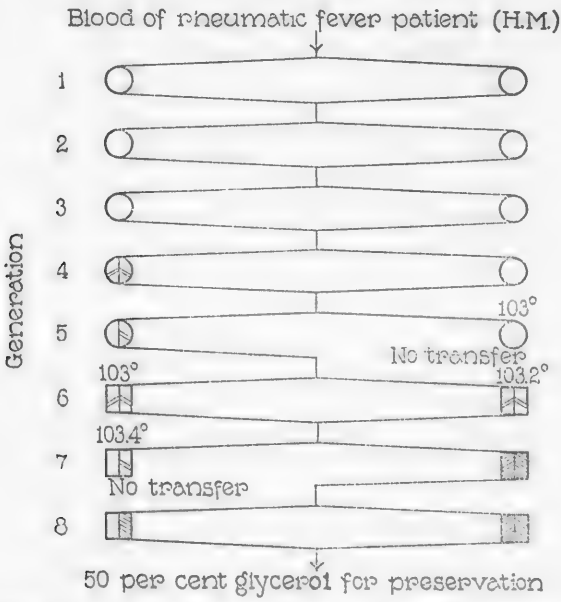
Rheumatic Fever Series.

Patient furnishing material.	Material used for inoculation.	No. of generations rabbits received benzene or x-ray.	Interval between transfers.	No. of generations transfers were carried on.	Generation in which definite evidence of infection appeared.
			days		
J. R.	Throat washings.	—	10-14	4	—
"	Joint fluid.	—	10–24 (mostly 14).	6	—
"	" "	—	8–23	4	—
M. C.	" "	Benzene, 6.	9–12	6	—
"	" "	X-ray, 3.	7-9	3	—
E. R.	" "	Benzene, 8.	7–9	8	5th
N. F. E. R.	Blood.	" 11.	4	23	4th
F. C.	"	" 5.	4	5	—
E.H.	"	" 5.	4	7	—
J. C.	"	" 9.	4	14	6th
"	"	" 7.	4	7	—
H. M.	"	" 5.	4	8	7th

As the rabbits were usually killed on the 5th day after inoculation, few data are available as to the duration of the fever and other symptoms. One rabbit, however, which was inoculated into both testicles and castrated on one side on the 5th day, continued to have fever of about 104° for 12 days after inoculation; but fever of this duration was exceptional.

The gross appearance of infected testicles varied. Some were greatly swollen and purplish red in color, with much distended capsules. On section there was oozing of serum; the testicular paren-

chyma was soft and showed at times yellowish necrotic areas, like small abscesses, scattered through its substance. Animals with such acutely infected testicles usually had high temperatures; and



TEXT-FIG. 1. The occurrence of clinical and histological evidences of orchitis in the rabbits of one series originally inoculated with rheumatic fever blood. All transfers made in duplicate, as shown, at 4 or 5 day intervals. In all rabbits with fever over 103°F., the maximum temperature is shown. All testicles were cultured at the time of transfer; all cultures remained sterile.

the disease in them appeared to have a shorter incubation period (2 or 3 days). In most cases the testes were only moderately swollen, congested, and soft. In still other instances there were no macroscopic changes, even though microscopic examination showed histological lesions. In one series of rabbits an apparent variation in virulence went in waves of 2 or 3 generations with acute hemorrhagic lesions followed by 2 or 3 which were macroscopically almost normal; this sequence was repeated three times without obvious explanation. The occasional deaths among our rabbits could always be attributed to some cause other than this infection.

Pathological Description.—Microscopic examination of sections of severely affected testicles stained with eosin and methylene blue revealed a diffuse orchitis (see Fig. 1) with "nuclear inclusions."

The seminiferous tubules contained no spermatozoa, the germinal epithelium was morbidly altered, often necrotic. The intertubular spaces were filled with inflammatory cells; the predominant type of cell had a large, pale, irregularly elliptical nucleus and pale or bluish pink cytoplasm and corresponded in appearance to endothelial leucocytes, or macrophages. Polymorphonuclear neutrophils and eosinophils and plasma cells occurred in variable numbers. The most characteristic feature was the presence in the nuclei of certain cells of uniformly stained pink or purplish bodies, sometimes multiple, but usually single, and always surrounded by a clear zone (see Fig. 2). They varied from tiny granules to large bodies which seemed to distend the nucleus to unusual size. The only blue-staining chromatin material in such swollen nuclei was a thin rim enclosing the narrow clear zone surrounding the inclusion body. These nuclear inclusions occurred in great numbers in some testicles, and were usually obvious enough to be easily discernible with the low power objective of the microscope. The alteration in the morphology of the cells containing inclusion bodies made difficult a diagnosis of their type; but whenever this was possible the cells appeared to be endothelial leucocytes. Nuclear inclusion bodies were occasionally found in testicles showing relatively little abnormality. Small areas of hemorrhage and edema were often present, and engorgement of the smaller vessels was common.

Sections of testicles which had been infected for 2 or 3 weeks showed more or less complete destruction of the germinal epithelium, fewer inflammatory cells in the intertubular spaces, and proliferation of fibroblasts and newly formed capillaries. Such sections never contained nuclear inclusion bodies; in fact these were never found later than 10 days after inoculation of the testis. The picture described above is that of a well marked infection. The changes were not always diffuse; spermatogenesis was sometimes comparatively little disturbed.

Skin Inoculations.—Successful attempts were made to inoculate the virus into organs or tissues other than the testicle.

After one or two unsatisfactory attempts to produce lesions by rubbing testicular emulsion into the scarified skin of rabbits, the intradermal method of inoculation employed by Rivers and Tillett (6) was adopted and gave reliable results. The rabbits were clipped and shaved on the flank and 0.2 cc. of testicular suspension was injected intradermally with a fine needle. The emulsion used for this purpose was thinner than that used for testicular inoculations; it was usually a 10 or 20 per cent suspension centrifuged for 5 minutes at high speed. After such inoculations an erythema appeared on the 3rd to 6th day, and increased in intensity for 2 or 3 days to an area of from 2 to 3 cm. in diameter slightly raised above the surface; after this it gradually faded. A few days later the shaved areas of the skin of the rabbits often became pigmented, while the previously erythematous areas remained pale. Histological examination of the infected skin showed that the lesions were immediately below the epidermis; they consisted of infiltration with mononuclear cells, mostly endothelial leucocytes, also variable numbers of lymphocytes and polymorphonuclears. Nuclear inclusions like those found in the testicle were not numerous but a few typical ones could usually be found on careful search (see Fig. 3). Suspensions of normal testicles produced no lesions like those described above, either macroscopically or microscopically.

Intrathoracic Inoculations.—At a time when the relation of the virus to rheumatic fever was still an open question, six rabbits were inoculated with testicular suspension into the right side of the chest, in the neighborhood of the heart. One of these died during the inoculation; three of the other five developed a fibrinous pericarditis.

In two rabbits this was limited to the parietal pericardium near the site of injection; although the pleura appeared normal in these rabbits, small masses of fibrin were found free in the pleural cavity. Histological examination showed the pericardium to be extensively involved in some areas and comparatively normal elsewhere. In the more severely affected zones the lining endothelial cells were swollen, cuboidal or columnar, and in places piled one on the other in a thickness of two to four cells. Slightly deeper in the pericardium there were marked accumulations of cells of various types, such as endothelioid cells, lymphocytes, a few fibroblasts, and occasional polymorphonuclear eosinophils. A few multinuclear cells were seen with closely packed nuclei. Pink-staining nuclear inclusion bodies similar to those seen in the testicle and skin were not infrequently encountered (see Fig. 4). Beneath the most actively inflamed visceral pericardium an occasional yellowish area was seen passing into the myocardium. Histologically there were myocardial lesions of various sizes and types. In places radiating from foci of pericarditis such as described above there were strands of mononuclear cells

passing out between the muscle fibers for variable distances. Elsewhere small lesions were encountered not in connection with an inflamed pericardium; these consisted of small clumps of mononuclear cells of various types closely packed together, and from them passed columns of similar cells radiating out between muscle fibers. One lesion was seen about 1 mm. in diameter near the base of the left ventricle, not far from the endocardium. Microscopically it consisted of a mixture of irregularly shaped degenerating muscle cells, a few wandering cells, and many proliferating cells. The latter were elongated, often branching, and had long oval vesicular nuclei; in places they were very large and had several nuclei arranged in rosettes; in other places multiple nuclei were arranged in rows. These giant cells have been described in various types of myocarditis, and are supposed to arise from the degenerating muscle fibers. At the periphery of the lesion the degeneration of muscle gradually decreased in intensity until the pathological appearance consisted simply of more or less normal appearing muscle fibers separated by a few mononuclear cells. The endocardium near this lesion had an appearance similar to that seen in the foci of pericarditis. Nuclear inclusion bodies were found both in the markedly involved area and between the normal appearing muscle cells at the periphery of the lesion (see Fig. 5). That these changes were not due merely to the irritation of the testicular suspensions is indicated by the results of eight earlier intrathoracic injections. These were made with material from a series which remained quite negative; in no instance was a fibrinous pericarditis or a myocarditis produced.

Intracerebral Inoculations.—Two fully etherized rabbits were inoculated intracerebrally through trephine holes over the left frontal lobe of the brain with 0.25 cc. of testicular emulsion. From one of these animals a series was carried on with transfers every 4 days for 4 generations.

In no case was clinical evidence of encephalitis forthcoming; nor were characteristic changes found on microscopical examination. At most there was a little fever, usually during the 2 days following inoculation. While the majority of the rabbits were sacrificed on the 5th day in the hope of carrying on infection, two were allowed to survive for 18 and 20 days respectively, but remained unaffected. The data are, however, insufficient to establish that rabbits cannot be infected by intracerebral inoculation especially in view of the finding of Rivers and Tillett (6) that rabbits can be immunized by that route against the virus they studied.

Immunity Phenomena.—It was found that rabbits inoculated with the virus either into testicles or skin were refractory to further intradermal inoculations when tested between 12 and 20 days later. No detailed experiments were conducted to determine how soon the

immunity appeared or how long it persisted. Rabbits immunized against one strain of virus were later immune both to the same strain and to other strains with which we were working. The results of our experiments are shown in Table II. The virus used in testing for immunity was in every case shown to be active by parallel tests on uninoculated rabbits.

In order to obtain further evidence as to whether a true immunity was present, the power of the serum of inoculated animals to neutralize the virus *in vitro* was tested. The testicular suspension used for skin inoculation on a normal rabbit was previously mixed with an equal volume of the serum to be tested; control inoculations of suspension mixed with an equal volume of saline or normal rabbit serum were made on the same animal. Each test was run in duplicate. It was found that sera taken between 15 and 21 days after the inoculation of a rabbit possessed the power of neutralizing all strains of the virus *in vitro*. Details of the experiments are shown in Table III.

Relationship of the Virus to That Described by Rivers and Tillett.—At an early stage in the investigation it became obvious that the virus with which we were working resembled in many ways that encountered by Rivers and Tillett (6, 7) in the course of their studies on chicken-pox. The technique employed by us was identical with theirs; our virus gave similar clinical, macroscopic, and histological pictures in rabbits, and similar skin lesions. Immunologically, also, it behaved in the same way. The only difference was that their rabbits had been originally inoculated with the blood of chicken-pox patients, while ours had received the blood or joint fluid of patients with acute rheumatic fever. Cross-immunity experiments were accordingly conducted in cooperation with Dr. Rivers and Dr. Tillett in order to elucidate the question of the relationship between the two viruses. These are also summarized in Tables II and III. The experiments showed that animals inoculated with our virus were later refractory to skin inoculations with theirs and *vice versa;* serum neutralization tests confirmed the idea that the two viruses were identical. It may be mentioned that Rivers and Tillett (6) had on other grounds begun to doubt the etiological relationship of their virus to chicken-pox.

TABLE II.

Results of Intradermal Tests for Immunity.

Rabbit No.	Original inoculation.			Period between inoculations.	Second inoculation.	
	Virus used.	Site.	Reaction.		Virus used.	Reaction.
				days		
1	FR	Testis.	? 0	29	Rivers-Tillett III.	0
2	"	"	? 0	29	" III.	0
3	"	"	? +	15	FR	0
					Rivers-Tillett III.	0
4	"	"	? +	15	FR	0
					Rivers-Tillett III.	0
5	"	"	? +	15	FR	0
					Rivers-Tillett III.	0
6	"	"	? +	16	CA	0
7	"	"	+	12	"	0
8	"	Skin.	+	12	"	0
9	"	"	+	12	"	0
10	"	"	+	20	FR	0
					CA	0
11	CA	"	+	15	Rivers-Tillett III.	0
12	"	"	+	15	" III.	0
13	"	" and testis.	+	20	FR	0
					CA	0
14	"	" " "	+	20	FR	0
					CA	0
15	"	Testis.	+	14	FR	0
					CA	0
16	"	"	+	14	FR	0
					CA	0
17	"	"	+	14	Rivers-Tillett III.	0

TABLE II—*Concluded.*

Rabbit No.	Original inoculation.			Period between inoculations.	Second inoculation.	
	Virus used.	Site.	Reaction.		Virus used.	Reaction.
				days		
18	CA	Testis.	+	14	Rivers-Tillett III.	0
19	Rivers-Tillett III.	Skin.	+	28	FR	0
					CA	0
20	" "	"	+	29	FR	0
					CA	0

TABLE III.

Results of Serum Neutralization Tests.

Rabbit No.	Tissue inoculated.	Virus.	Time between inoculation and bleeding.	Neutralizing power of serum.	For what virus.	No. of rabbits tested on.
			days			
3	Testis.	FR	21	+	FR	2
4	"	"	21	±	"	2
5	"	"	21	+	"	2
	"	"	21	+	CA	1
	"	"	21	+	HM	2
6	"	"	21	+	FR	2
11	Skin.	CA	15	+	"	2
				+	CA	1
12	"	"	15	±	FR	2
20	"	Rivers-Tillett III.	4 wks.	+	CA	1
21	?	" "	14 (over).	+	"	1
22	Uninoculated.	—	—	+	FR	2
23(*a*)	"	—	—	—	"	2
(*b*)	Skin.	FR	14	+	"	1
24	Uninoculated.	—	—	—	"	1

A still more conclusive experiment was performed later with two rabbits. These were bled and their serum was found not to neutralize our virus; they were then inoculated both intradermally and intratesticularly with our virus. The skin inoculations took well; the testicles were inflamed, and one of them removed after 4 days, under ether anesthesia, showed characteristic nuclear inclusions in sections. 23 days after inoculation the rabbits were bled; their serum was found to neutralize our virus, and the rabbits were found to be refractory to intradermal inoculation with the Rivers-Tillett virus.

In Vitro Neutralization Tests with Rheumatic Fever Sera.—In order to determine the relationship of this virus to rheumatic fever, similar neutralization tests were performed with the sera of eleven rheumatic fever patients, and with that of three patients with cardiac disease, probably of rheumatic origin. Some of the rheumatic fever patients were convalescent, but in most cases they showed evidence of persisting infection. In no instance was there any evidence of neutralization of the virus by these sera. These findings confirmed our growing suspicion that the virus isolated bore no relation to rheumatic fever.

Survival of the Virus in Vitro.—Attempts were made to preserve the virus *in vitro* in two ways: (1) by the aid of glycerol; and (2) by drying the virus in a frozen state.

1. Glycerol.—Active testicular emulsions were briefly centrifuged; the supernatant was well mixed within an equal volume of pure glycerol, placed in a tube sealed with paraffin, and kept in the ice box.

This was tested as follows: Some active emulsion of testicles infected with Virus FR, after storing with glycerol up to 50 per cent for 10 days, caused acute orchitis in one rabbit. Two more samples stored for 14 and 18 days respectively were proved active in the same way. (See Table IV for details of experiments.)

2. Freezing and Drying.—Swift (8) has published the details of a technique for preserving stock bacterial cultures by freezing and drying them in the frozen state. He found that bacteria of various types could be preserved for years in this manner; and quotes observations showing that the viruses of rabies and of Rous' chicken sarcoma can be preserved in the same way. In this way we stored active testicular emulsions from several rabbits; the results of the subsequent tests are shown in Table IV.

In this table, a positive intratesticular inoculation means the production of an orchitis with the presence of nuclear inclusions on histological examination.

It will be seen that the frozen and dried virus readily survived for a few days, but after 10 weeks had apparently lost some or all of its activity. It is unlikely that the positive results after one transfer can be ascribed to our picking up the virus anew, since in none of our other series nor in eleven of Rivers and Tillett's did a virus ever appear earlier than the 4th generation.

TABLE IV.

Table Showing Results of Attempts to Preserve the Virus.

Strain.	How preserved.	How long preserved.	Evidence of survival.	No. of rabbits tested on.
		days		
FR	In 50 per cent glycerol.	10	Positive intratesticular inoculation.	1
"	" 50 " " "	14	" " "	2
"	" 50 " " "	18	" " "	2
"	Freezing and drying.	5	" " "	2
"	" " "	10 wks.	Intratesticular inoculations apparently negative at first, but positive after one transfer.	2
CA	" " "	8	Intratesticular inoculation negative; but positive intradermal test after one transfer.	3
"	" " " (same batch).	10 wks.	No evidence of survival even after one transfer.	2

SUMMARY.

1. Blood and joint fluid of patients suffering from rheumatic fever were inoculated into the testicles of rabbits, and transfers were made from testicle to testicle at varying intervals. In three series, in none of which were the periods between the transfers longer than 4 days, a virus was recovered which produced acute orchitis in rabbits. The virus could be indefinitely propagated from rabbit to rabbit.

2. Intradermal inoculation of the virus led to the appearance of raised erythematous lesions in 3 to 6 days.

3. Intrathoracic inoculations near the heart led to a fibrinous pericarditis in three out of five rabbits, and to a myocarditis in one.

4. Nuclear inclusion bodies staining pink with eosin were found in the lesions in the testis, skin, pericardium, and heart muscle.

5. Rabbits inoculated into skin or testes were refractory, 2 weeks later, to further intradermal inoculations; after this interval their serum had developed the power to neutralize the virus *in vitro*.

6. The virus can be preserved in 50 per cent glycerol for at least 18 days. It can be preserved after freezing and drying for at least 8 days and probably for 10 weeks.

7. Cross-immunity tests point to the identity of the virus with that described by Rivers and Tillett in their studies on chicken-pox as Virus III.

8. These facts together with the failure of rheumatic fever sera to neutralize the virus indicate that it bears no etiologic relationship to rheumatic fever.

The authors desire to acknowledge their gratitude to Dr. Rivers and Dr. Tillett for their kind cooperation in conducting the cross-immunity experiments.

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EXPLANATION OF PLATES.

PLATE 34.

FIG. 1. Acute orchitis in the rabbit due to the virus described. Note intense infiltration of interstitial tissue with inflammatory cells of various types and with red blood cells; also marked degeneration of germinal epithelium. × 120.

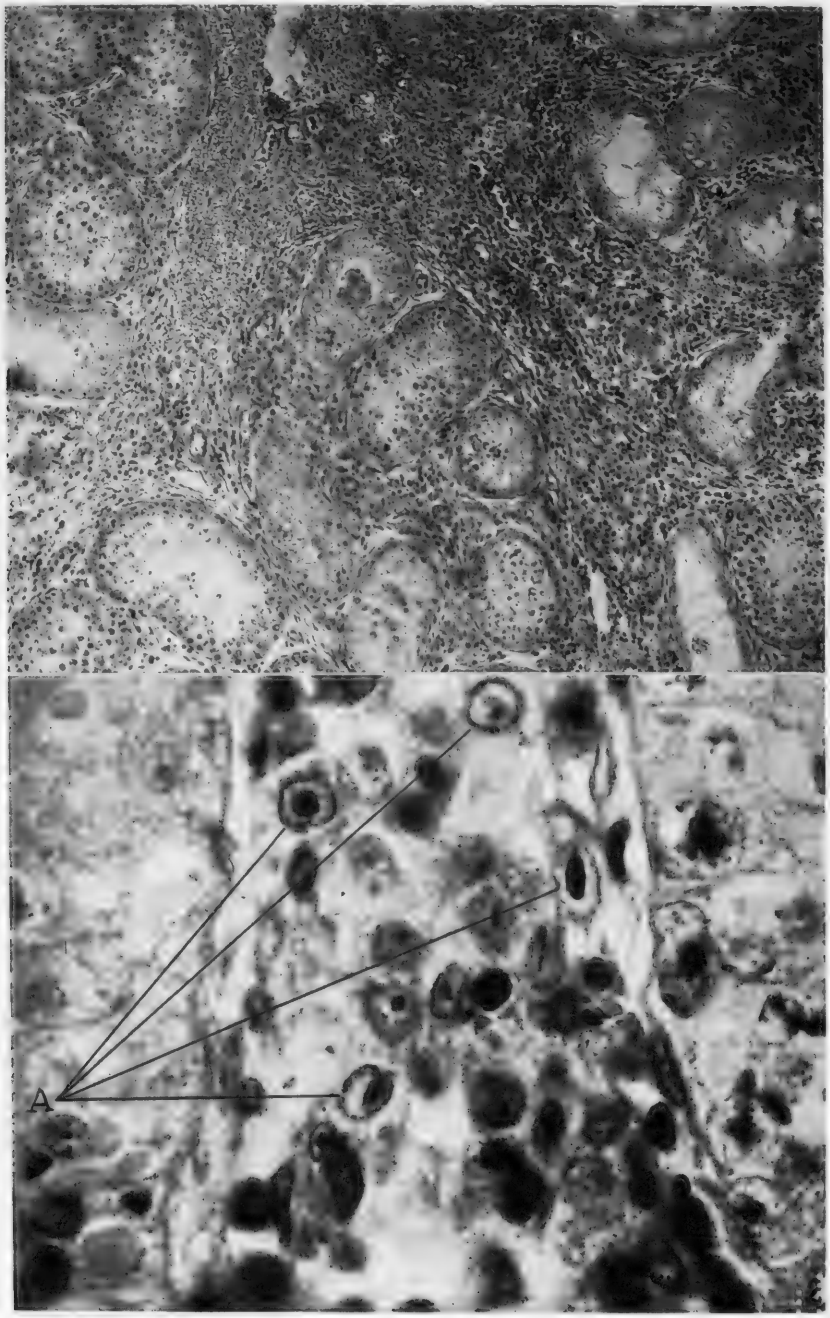
FIG. 2. The same on a larger scale, showing the type of cellular reaction and several nuclear inclusions (*A*); these stained pink with eosin and are seen in the middle of swollen vacuolated nuclei. The degenerated germinal epithelium is seen on either side of the interstitial tissue. × 1,100.

PLATE 35.

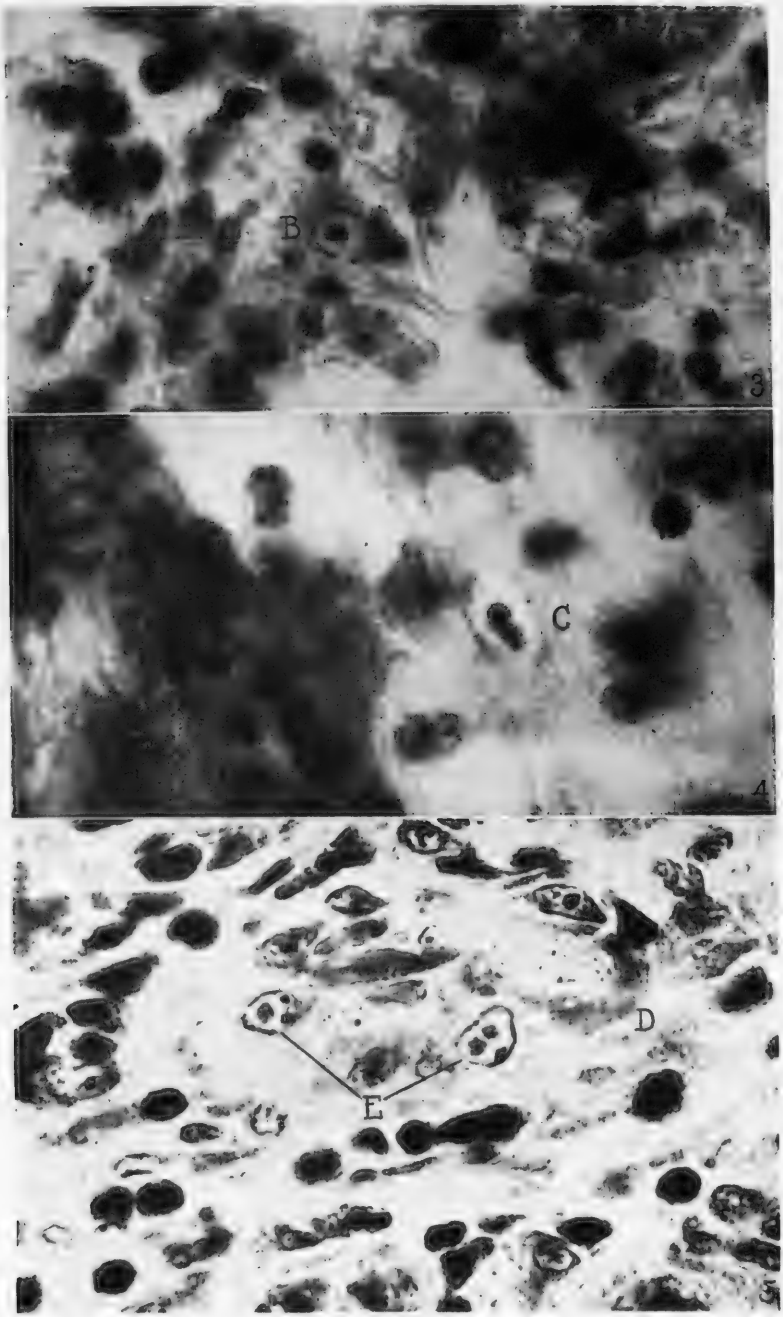
FIG. 3. Type of cellular reaction in a skin lesion. One nuclear inclusion (*B*) near the middle of the figure. × 1,100.

FIG. 4. Type of cellular reaction in a lesion in the pericardium. One nuclear inclusion (*C*) is shown. × 1,100.

FIG. 5. Type of cellular reaction in a myocardial lesion. Note degenerated muscle fiber (*D*) and two cells (*E*), which show one or two very dark nucleoli at the side, and rather paler nuclear inclusions at the center. × 1,100.



(Miller, Andrewes, and Swift: Filterable virus infection of rabbits. I.)



(Miller, Andrewes, and Swift: Filterable virus infection of rabbits. I.)

A FILTERABLE VIRUS INFECTION OF RABBITS.

II. Its Occurrence in Apparently Normal Rabbits.

By C. H. ANDREWES, M.D., and C. PHILIP MILLER, Jr., M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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INTRODUCTION.

In a preceding communication (1) we have reported with Dr. Swift the occurrence of a virus infection in several series of rabbits originally inoculated with material from patients with acute rheumatic fever. The macroscopic and microscopic evidences of infection of the rabbits and the immunological behaviour of the virus were described. Evidence was finally obtained to show that the virus was identical with that described as Virus III by Rivers and Tillett (2, 3), who were studying chicken-pox.

Since two groups of workers, studying chicken-pox and rheumatic fever respectively, had encountered what was apparently the same virus, it seemed likely that that virus bore no etiologic relationship to either disease; and this view has been borne out by the results of neutralization tests (3, 1). The phenomena encountered seemed best explained by the hypothesis that we were dealing with an organism of the filterable virus group which was pathogenic for the rabbit. Its source remained obscure. Several possibilities suggested themselves. (1) The virus might be circulating in the blood of normal human beings or in that of patients febrile from any cause, just as it is suggested that the herpes virus may occur in the cerebrospinal fluid of patients with encephalitis lethargica. (2) The etiologic agent of either chicken-pox or rheumatic fever might have accidentally made its way into the laboratories of workers with the other disease. (3) A contaminant might have been introduced from human respiratory passages or elsewhere. (4) We might be dealing with a virus of rabbit origin.

Methods.

Precautions against Contamination.—Control experiments were conducted in order to eliminate some of these possibilities. Six series of rabbits were injected as in the previous work, but the human element was excluded by using rabbit blood as the original inoculum. Before this experiment was performed certain precautions were taken; for Rivers and Tillett (3) have obtained evidence suggesting that cage infection of rabbits with the virus is possible. On at least two occasions they noted that when one rabbit in a cage was refractory to their virus, others in that cage proved also refractory. Moreover, dropping some infected testicular suspension into a rabbit's nose was shown by them to render that animal immune. It was therefore necessary to exclude all possibility of infection of our new rabbit stock either from our original virus strains or from the virus with which Rivers and Tillett were still working in another building of the Hospital. Accordingly, all our animals were killed and the virus preserved in glycerol. Our animal room and laboratory were thoroughly cleansed with disinfectants in case any virus should be lurking about the premises. Precautions were taken that no person came in contact both with our animals and with those of Rivers and Tillett. Thus as far as possible all opportunity for cross-contamination was eliminated.

Six series of rabbits were inoculated into the testicles with the fresh blood of apparently normal rabbits. Otherwise the technique employed was the same as in the original experiments. Every 5th day, as before, the animals were sacrificed and the testicles of one or both of each pair inoculated into another pair of rabbits. In three series the rabbits were treated with benzene before inoculation, for 4, 6, and 7 generations respectively. The other three series received no benzene. In each case the testes were cultured and examined histologically as described earlier. It was obviously possible that if one series developed an infection with the virus, the other series might become infected from it. Hence one positive series would be of as much value as several. Nevertheless, we tried to diminish any possibility of cross-infection by keeping a set of cages and a separate thermometer for each series.

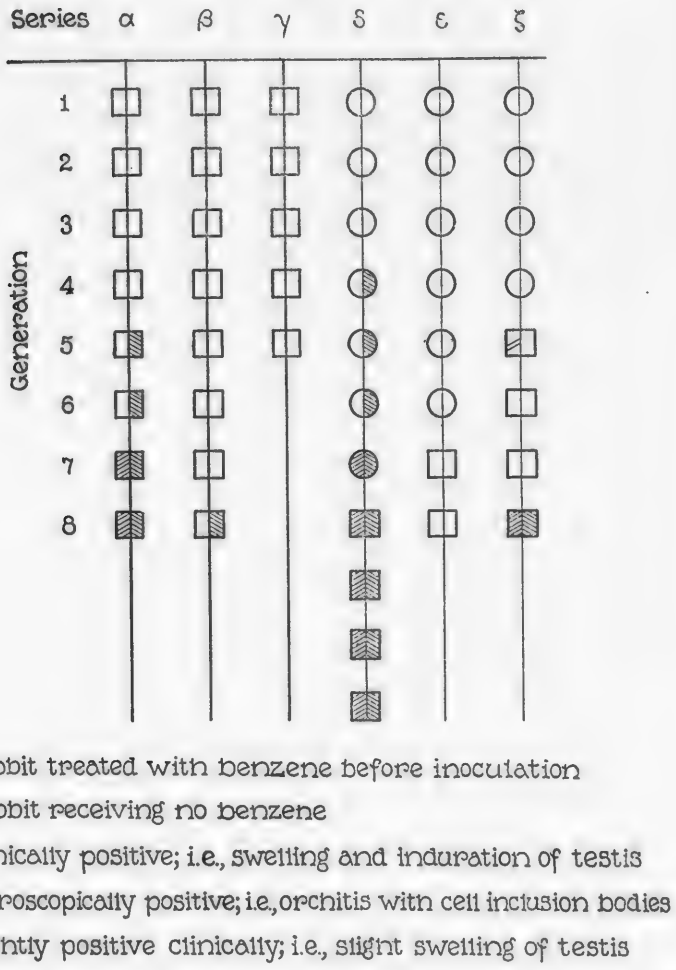
RESULTS.

The results were striking. For the first 3 generations no rabbit showed clinical or histological evidence of infection The 4th generation of one of the three benzene-treated series yielded histological evidence of infection, with typical nuclear inclusions. The infection was then readily carried on up to the 11th generation, when the virus was preserved in glycerol. Macroscopical evidence that the series was positive appeared by the 6th generation. One of the series not treated with benzene showed in the 5th generation a typical histological picture with nuclear inclusions; and after one further transfer it was positive clinically. This virus was carried on up to the 8th generation, when it was stored. The clinical, macroscopical, and histological pictures were identical in every way with those encountered before, except that fever occurred only occasionally and the testicles were never quite so acutely inflamed as in some rabbits infected with the original strains reported in our first communication (1).

The immunological identity of the two new strains with our original strains and that of Rivers and Tillett was established as follows: Two rabbits infected in both skin and testes with one of the new strains were refractory 17 days later to the Rivers-Tillett III virus inoculated intradermally. One rabbit inoculated into skin and testes with the other new strain was immune 14 days later to Rivers-Tillett Virus III. Each of the new strains was tested intracutaneously on two rabbits and was found to be neutralized *in vitro* by the serum of a rabbit immunized with our original FR strain; this serum was known to neutralize the homologous virus. We therefore felt convinced that the virus isolated in the two control series was identical with strains originally encountered by Rivers and Tillett and by us.

In view of the possibility of cross-infection, less emphasis can be laid on the fact that two more of the six control series (one treated with benzene and one not) showed nuclear inclusions in their testes in the 8th generation. No steps were taken to establish definitely that these changes were caused by the virus under study.

The results of the control series inoculations are shown in the text-figure.



TEXT-FIG. 1. The occurrence of clinical and histological evidences of orchitis in six control series, three treated with benzene, three not so treated. All transfers made at 4 or 5 day intervals. All inoculations made in duplicate; *i.e.*, each generation in each series consisted of a pair of rabbits.

DISCUSSION.

There seems no doubt that in the course of our experiments an active transmissible agent has been encountered. This has been referred to, for the sake of simplicity, as a virus. There is, however, no absolute certainty that it is a living organism. We have been unable to demonstrate a bacterium, spirochete, or indeed anything visible under the microscope which seems likely to be a parasite. We failed in two attempts to pass the active agent through Berkefeld V filters; but this has been accomplished by Rivers and Tillett (3), working with what we believe to be the same virus. The immunological behaviour of the agent is strongly suggestive of its being a living organism; it is comparable with that encountered in poliomyelitis (4). The fact that the agent can apparently be propagated indefinitely indicates that it is probably living, or at any rate that it can reproduce itself indefinitely and give rise to characteristic reactions under the conditions of the experiment. Above all, the appearance of the nuclear inclusions, which are so very similar to those encountered in the lesions of the various forms of herpes and of chicken-pox in man, makes it almost certain that the virus belongs to the same class as the causative agents of those diseases. It is impossible to enter here into a discussion of the significance of the nuclear inclusion bodies. The weight of evidence seems to be in favour of Lipschütz's (5) view that such inclusion bodies represent a specific reaction on the part of the nucleus to certain viruses; but it is not easy to say whether or no the virus causing these changes is contained within the inclusion body.

The nature and source of the virus are of great interest. It seems certain that animals inoculated with active material are refractory a fortnight later to intracutaneous inoculation with the same material; also that their serum then possesses the power of neutralizing the virus *in vitro*. By making use of these reactions it has been possible to demonstrate that the different strains of virus isolated in both the rheumatic fever series and the control series are identical with each other and with Virus III of Rivers and Tillett. In drawing this conclusion we have not lost sight of the facts that Rivers and Tillett (3) have found that 15 per cent of young stock rabbits seem to be naturally refractory to skin inoculation with their virus and that the

serum of 20 per cent of uninoculated stock rabbits of different ages will neutralize the virus. We ourselves encountered one rabbit out of five the serum of which possessed this property.

The results of the control series appear to exclude the possibility that the virus takes its origin from normal or abnormal human blood. The same experiments, taken together with the failure of rheumatic fever sera to neutralize the virus, make it highly improbable that the latter is related to rheumatic fever. A similar conclusion applies to chicken-pox with the reservation that since Rivers and Tillett had been working with their virus before we adopted exactly the same technique, it is possible, but highly improbable, that a chicken-pox virus could have been accidentally introduced into our animal house and never eliminated in spite of all our precautions. An accidental contamination from human respiratory passages or elsewhere cannot be certainly excluded, but it is improbable because this virus has not apparently been recognized except during the employment of the technique described.

A number of facts point to the rabbit as the source of the virus. It has appeared in at least two series of rabbits wherein no human material was used for inoculation. The facts that a certain proportion of rabbits are naturally refractory and that their serum neutralizes the virus *in vitro* suggest spontaneous infection and recovery with the development of a demonstrable immunity.

If the simplest interpretation that the virus is a spontaneous rabbit parasite, be accepted, a number of interesting problems are raised. What organs does it involve? How is it naturally transmitted? Some of Rivers and Tillett's observations suggest that the virus tends to localize in the skin which has been irritated by shaving, but these observations were made under the artificial conditions of experimentation. Since rabbits quickly become immune following experimental infection, it would seem that in order to pick up the virus in the course of transfers, it would be necessary to encounter a rabbit in the early stages of the spontaneous infection, unless of course this natural infection and its resulting immunity have a different evolution. And yet both Rivers and Tillett and ourselves were able to recover and carry along the virus with remarkable regularity in about 50 per cent of our series. Moreover, the infection usually

appeared between the 4th and 7th generations. That it never appeared before the 4th generation is noteworthy and perhaps indicates that the quantity of virus or its virulence must be increased by one or two passages before it can be recognized. The treatment of rabbits with benzene apparently had no effect on the ease with which the virus was found. This is shown both by comparing the two sets of control series, and also by comparing our results with those of Rivers and Tillett.

It seems, then, fair to assume that a virus has been met with both by Rivers and Tillett and by ourselves which has not previously been described, and which is probably of rabbit origin. If this is true it is of importance from several points of view. First, a virus is at hand which can be readily studied in a common laboratory animal in which it spontaneously occurs. Second, as the rabbit testis is used as a method for propagating and purifying vaccine virus (6), there may be a danger of picking up and carrying along this virus at the same time, a point which needs further investigation; the danger could probably be avoided by using only animals naturally refractory to or artificially immunized against the supposed spontaneous virus. The inoculation of rabbit testes as a means of cultivating microorganisms is growing in favour. While it seems probable that the virus of symptomatic herpes causes similar changes in the rabbit testis and similar nuclear inclusion bodies to those described in these papers, yet much work on this and other viruses will have to be reexamined in the light of our present knowledge. Parker (7) has recently stated that intranuclear inclusion bodies in the rabbit can probably be considered specific for a herpetic infection; this statement must now be modified. In view of Goodpasture and Teague's (8) opposition to the theory of Levaditi (9) and others that the herpetic and allied viruses are strictly ectodermotropic, it is noteworthy that the virus under discussion readily infected myocardium and pericardium as well as testicle and skin. It exhibits, therefore, no exclusive affinity for structures of any one embryonic origin. Its ability to infect entoderm was not put to test. Finally, if the method of intratesticular transmission at short intervals is used in the search for unknown etiologic agents the presence of the virus described will have to be borne in mind and perhaps guarded against by previous immunizations.

SUMMARY.

1. In two, and possibly four, of six transmission series of rabbits inoculated in the beginning with the blood of apparently normal rabbits a virus was recovered having the same properties as that recovered from series originally inoculated with material from rheumatic fever patients. The virus was immunologically identical with Rivers-Tillett Virus III and with that obtained from the rheumatic fever series. In conducting the control series precautions were taken to prevent infection of rabbit stock from known sources of the virus.

2. Treating rabbits with benzene does not appreciably increase their susceptibility to infection with the virus.

3. The virus is probably a parasite of the domestic rabbit but up to the present time the natural course of the infection in that animal is unknown.

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STUDIES ON THE TOTAL BILE.

VII. Conditions Influencing the Calcium Content of the Bile.

By DOUGLAS R. DRURY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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The rôle of calcium salts is admittedly an important one in the formation of gall stones. There is no need to detail in this connection the large literature on calcium carbonate stones, which are the common calculi of the herbivora and of the dog,[1] or to recount the hypotheses that have been advanced to explain the occasional occurrence of carbonate stones in human beings and the more frequent calcium bilirubinate concretions. The precise sources of the calcium, the influences modifying its amount, and the factors responsible for its deposition from the bile have long been matters of debate. Certain aspects of the problems involved have been considered in previous communications from this laboratory.[1-3] The present paper is concerned with the total calcium output in the bile and the influences modifying it. Knowledge on these points is fundamental to an understanding of the genesis of calcium stones.

Hoppe-Seyler[4] has published analyses of the bladder and liver biles of dogs. He gives figures on the amounts of calcium present in a single instance,—41 mg. of calcium per 100 cc. of bladder bile and 31 mg. per 100 cc. in the liver bile. According to Jankau's more extensive observations, there exists between 13 and 17 mg. of calcium in every 100 cc. of fistula bile of the dog, an output unincreased by the administration of lime salts by mouth.[5] Lichtwitz and Bock[6] studied the calcium

[1] Rous, P., McMaster, P. D., and Drury, D. R., *J. Exp. Med.*, 1924, xxxix, 77.
[2] Rous, P., Drury, D. R., and McMaster, P. D., *J. Exp. Med.*, 1924, xxxix, 97.
[3] Drury, D. R., McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1924, xxxix, 403.
[4] Hoppe-Seyler, F., Physiologische Chemie, Berlin, 1878, pt. 2, 302.
[5] Jankau, L., *Arch. exp. Path. u. Pharmakol.*, 1891–92, xxix, 237.
[6] Lichtwitz, L., and Bock, F., *Deutsch. med. Woch.*, 1915, xli, 1215.

content of human bile obtained from patients with biliary fistulas, after operation. The amount of calcium varied from 4 mg. to 9 mg. per 100 cc. bile. The literature contains no observations upon the effect of normal influences on the calcium of the bile. The investigators above mentioned examined isolated samples of the secretion or specimens which represented but a small portion of the 24 hour output. The liver bile, collected as it was from exposed fistulas in which the gall bladder formed a link, was subject to the concentrating influence of this viscus[7] and to infection and must often have been contaminated with fluid derived from an inflamed duct system. For the purposes of the present work a method for the permanent intubation of the common duct has been employed whereby during weeks or months the entire yield of bile may be obtained, in a sterile condition, and from uninflamed bile passages.[8] The influence of the gall bladder was ruled out by severing its neck between ligatures.

The following method of determining the total calcium was employed.

Duplicate 5 or 10 cc. portions of the 24 hour bile were pipetted into platinum dishes, evaporated to dryness, and then ignited with the aid of a little nitric acid. The residue was dissolved with 2 or 3 drops of hydrochloric acid and taken up in about 40 cc. of water containing a drop of phenolsulfonphthalein solution. The solution was neutralized by adding enough ammonia to develop a slight pink color of the indicator. The mixture was boiled until the color began to turn again to yellow, owing to the evaporation of the ammonia, and 10 cc. of a saturated solution of ammonium oxalate was added. For about half a minute thereafter the mixture remained perfectly clear and then very fine crystals of calcium oxalate began to appear. The boiling was continued for a minute or so and further precipitation allowed to take place overnight at room temperature. The precipitate was collected on an asbestos filter mat and washed free of ammonium oxalate, after which the mat was transferred to a beaker and 10 cc. of 50 per cent sulfuric acid followed by 100 cc. of water was added. The whole was heated to boiling, the oxalic acid titrated with N/100 $KMnO_4$, and the amount of calcium in the sample calculated from the amount of $KMnO_4$ required.

This is a modification of Kramer and Howland's method.[9] It was not necessary to use the small volumes of reagents which they employed, because of the larger amounts of calcium dealt with. Shohl[10] has shown that calcium, under the conditions mentioned, is

[7] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.
[8] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.
[9] Kramer, B., and Howland, J. H., *J. Biol. Chem.*, 1920, xliii, 35.
[10] Shohl, A. T., *J. Biol. Chem.*, 1922, l, 527.

quantitatively precipitated as calcium oxalate even in the presence of phosphates and magnesiums and that correct values are obtainable by the volumetric method.

Normal Variations in the Calcium of the Bile.

The calcium output from the liver was followed in five dogs for periods ranging from 17 to 60 days following the operation for intubation of the common duct. Under ordinary circumstances, when the animal was eating well, the amount of the element excreted per cc. of bile varied little from day to day and from individual to individual (Charts 1 to 4), a constancy rendered the more striking by the large concomitant variations in the bilirubin output. In contrast to this finding, the total 24 hour yield of calcium varied enormously as would inevitably follow from the fact that the bile quantity is subject to large physiological variations from hour to hour or from day to day, whereas the calcium per cc. remains, as just stated, relatively unchanged.

Throughout the natural and induced vicissitudes of bile excretion by the animals, the total amounts of the secretion and of calcium tended to vary together. The correspondence was especially noteworthy when the bile volume underwent an abrupt and considerable increase as when food had been given after a period of fasting or low intake (Charts 1,*B*, 2,*A*, and 4,*B*). When the bile amount diminished greatly below the normal average, as during prolonged fasts, there occurred a well marked increase in the concentration of calcium per cc. which compensated, in some part but far from wholly, for the lessened total amount of the vehicle of excretion (Charts 3,*B*, 4,*A*, 4,*C*, 6,*A*, and 7,*A*). Such compensation,—if one may use the term,—has been found to be practically perfect in the case of bile pigment,[11] every diminution or increase in the amount of bile being attended by a reciprocal increase or diminution in the concentration of pigment per cc., so that the total output for the 24 hours remains the same. The calcium concentration undergoes changes, upon food diminution or withdrawal, which are roughly similar to those of bilirubin, as the charts show (Charts 3,*B*, 4,*A*, 4,*C*, and 7,*A*), and this

[11] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

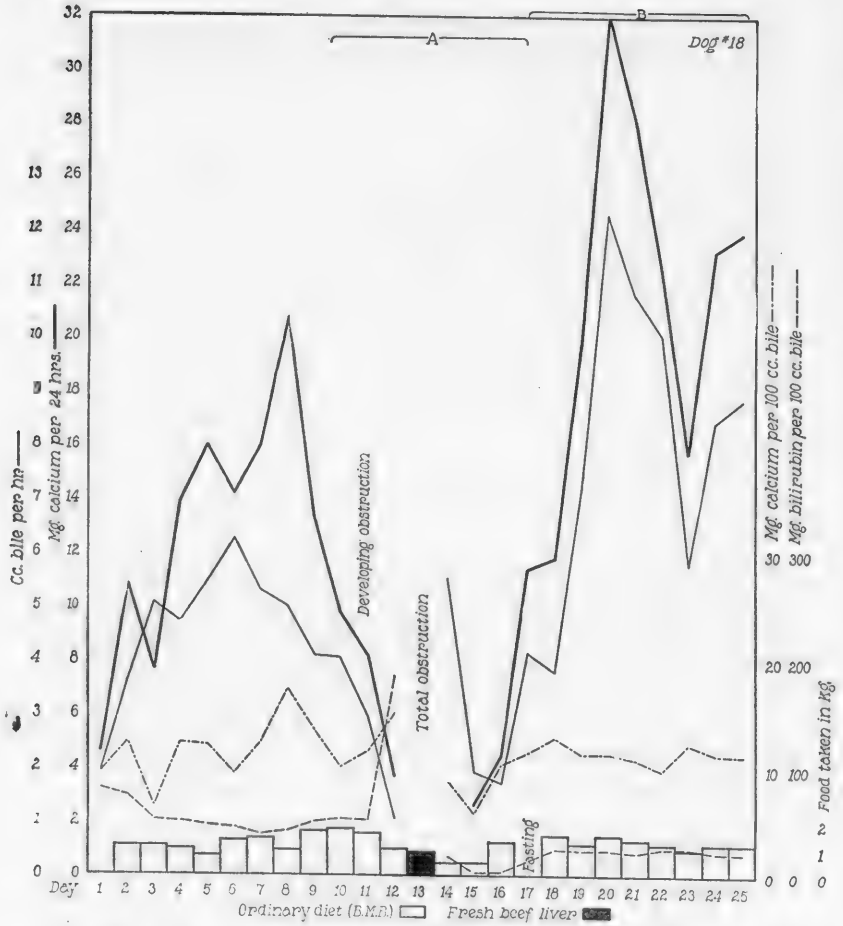

CHART 1. The chart shows (*a*) variations in the total bilirubin and bile calcium outputs such as ordinarily occur following intubation of an animal; (*b*) variations following the relief of an intercurrent obstruction to bile excretion. It will be observed that the calcium content per cc. of bile varied little from day to day despite the wide fluctuations in bile quantity. The importance of the phenomenon, as affecting the total calcium output, is especially noteworthy in the days immediately following relief of the obstruction, when the total amount of bile for the time being, enormously increased, and the total calcium, likewise increased, to approximately the same proportion. B. M. B. = beef, milk, bread.

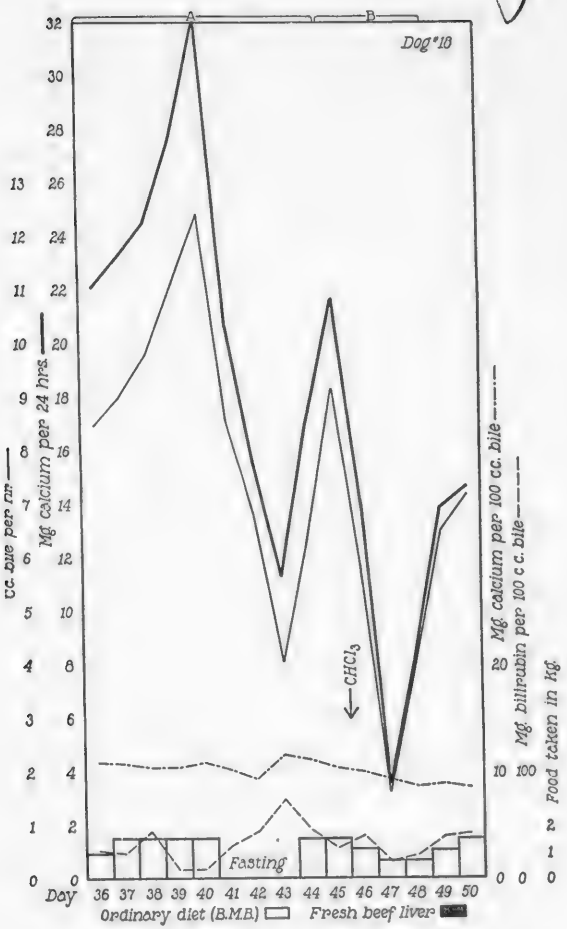

CHART 2. To show the constancy of the calcium output per cc. of bile and the consequent parallelism between the intercurrent changes in bile volume and total calcium output. The bilirubin output, by contrast, varied inversely with the total bile amount. On the 46th day of intubation the animal was anesthetized with chloroform for 2 hours. There followed a marked temporary drop in bile volume and calcium output, and, as ordinarily after such poison, some lessening in the bilirubin concentration.

is also the case when the volume of bile increases as when feeding is resumed. But the findings as a whole prove clearly that the total output of calcium toward the end of a fast of several days duration is well below the normal average.

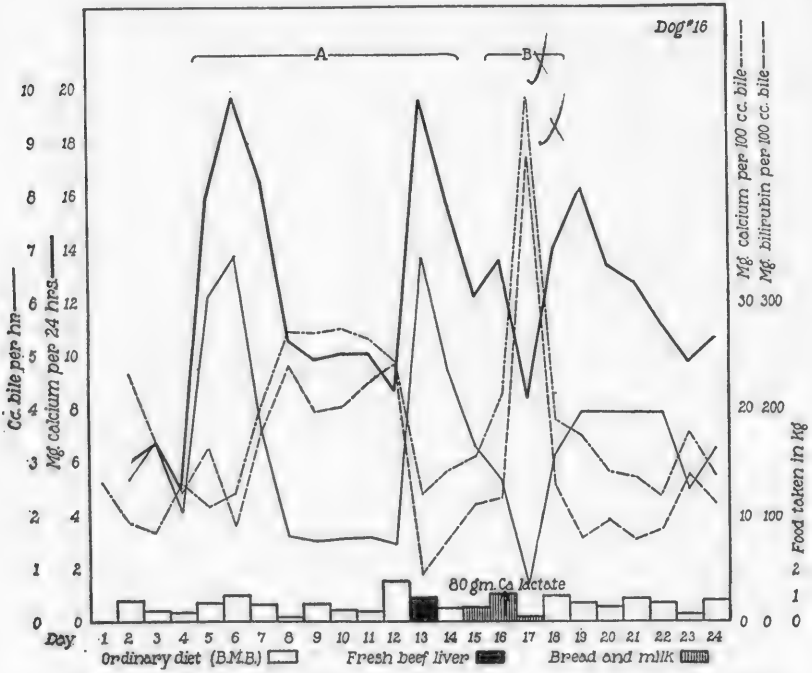

CHART 3. To show the variations consequent on altered food intake and calcium administration. During the first 4 days after intubation, the bile output was low as is the rule at this time. On the 5th to 7th days inclusive, much bile was put out and the total calcium output increased almost proportionately. Between the 8th and the 12th days the animal ate little. The bile volume became low as also the total calcium output, whereas concentrations of calcium and bilirubin became unusually high. On the 13th day a large feeding with beef liver resulted in an abrupt increase in bile volume. The administration of 80 gm. of calcium lactate by mouth on the 16th day was without demonstrable effect on the calcium excretion. The drop in bile volume and calcium output on the 17th day is attributable to the low food intake. The concentrations of calcium and bilirubin were greatly increased.

When there occurs a large fluid output from the liver, the concentration of calcium in the bile does not alter much, whereas that of bilirubin, as already stated, usually undergoes a diminution directly reciprocal to the increase in bile quantity. These facts are illustrated

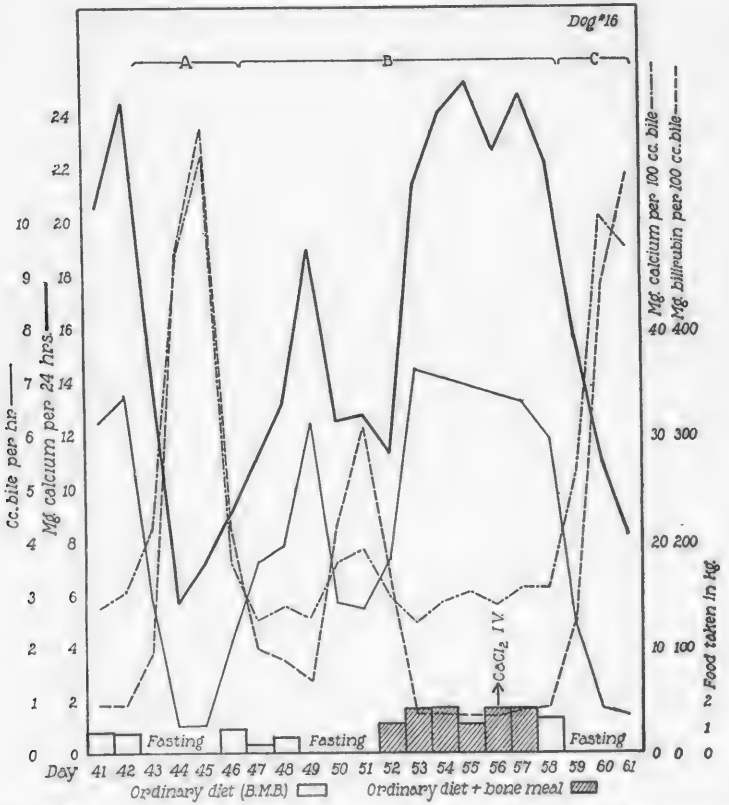

CHART 4. The changes in the bile resulting from fasting, feedings of bone meal, and an intravenous injection of calcium chloride. On the 56th day three intravenous injections of 10 cc. each of 10 per cent calcium chloride were given.

in Charts 2,*A* and 3. During a pronounced cholorrhea, the excretion of calcium per hour is strikingly increased. The greater the rise in the fluid output above the average, the more directly proportional to it does the calcium output become (Charts 1,*B*, 2,*A*, and 3,*A*).

Effect of Calcium Administration on the Calcium Content of the Bile.

The question whether variations in the lime salts of the diet influence the output of the element in the bile has long been under discussion. The effect of a diet very rich in them was tested on an animal the bile of which had been studied during many weeks (Dog 16). A preliminary fast of 3 days was instituted, extending from the 49th to the 51st day of intubation. On the 52nd day and thereafter until and includ-

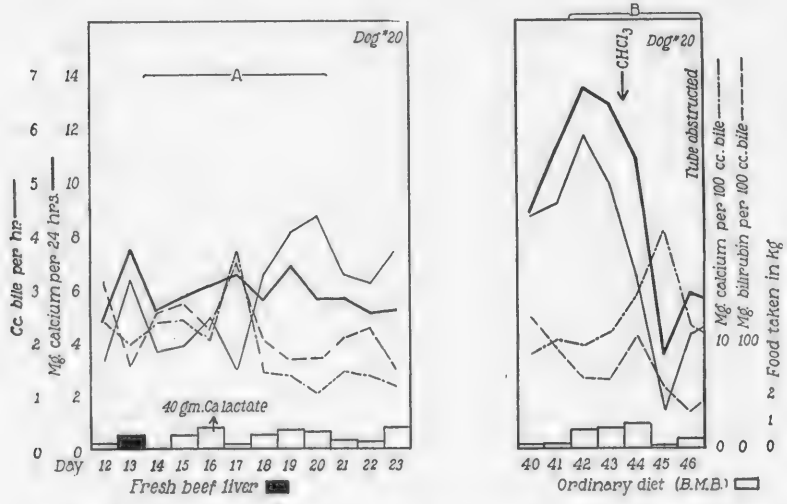

CHART 5. (*A*) Calcium lactate by mouth failed to alter the total calcium of the bile. The change in concentration is attributable to the lessened food intake. (*B*) Another instance of the changes following chloroform administration; anesthesia for 2 hours. An anomalous effect is to be seen in the temporarily heightened calcium concentration.

ing the 58th day, a diet was given consisting of 1,500 gm. of bread, milk, and lean meat in fixed proportion, plus 200 gm. of bone meal. The dog ate well of this mixture and always took over 1,000 gm. of it per day. The amounts eaten and the bile findings are given in Chart 4, *B*. As will be seen, the diet had effect in a large output of bile with the usual concomitant increase in calcium. But the increase is no greater than that on days when the animal put out the same quantity

of bile on a diet similar save that it lacked the bone meal, as Section *A* of Chart 3 shows. Evidently the changes in the calcium output were in this animal at least dependent rather on changes in the volume of the vehicle of excretion than on the presence of bone meal in the diet.

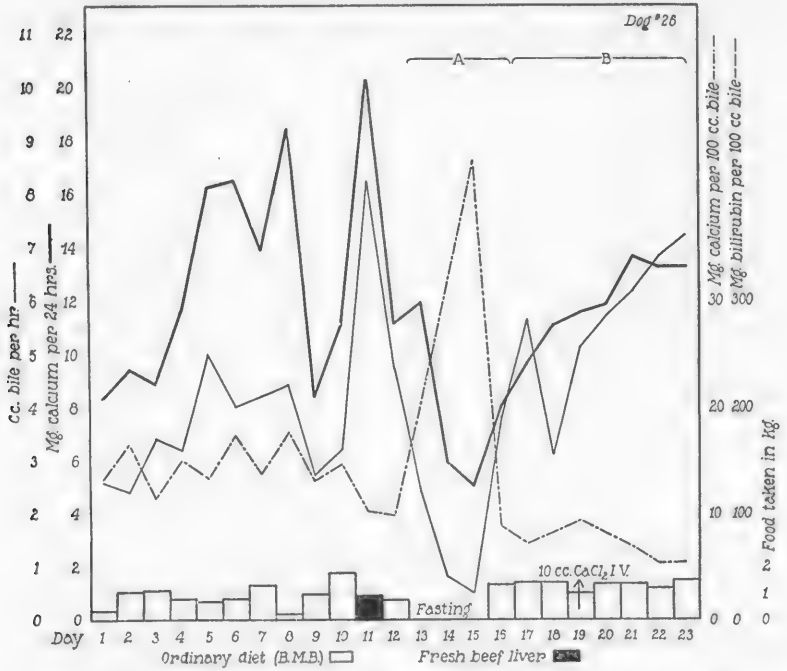

CHART 6. The effects of fasting, liver feeding, and the intravenous injection of a calcium salt on the character of the bile. On the 19th day three intravenous injections of 10 cc. each of 10 per cent calcium chloride were given.

Dogs 16 and 20 were given large doses (80 gm. and 40 gm.) of calcium lactate by stomach tube. The data as concerns the bile are recorded in Charts 3,*B* and 5,*A*. In the case of Dog 20, the addition of the salt to the diet had no observable effect on the calcium output from the liver. Dog 16 ate very little food the day after the dose of calcium was given and in consequence the output of bile was unusually

small. The calcium output fell markedly but did not differ in this particular from that of other dogs eating little and not fed lime salts (Charts 4,*A* and 6,*A*). There can be little doubt then that the administration of calcium lactate by mouth to dogs losing bile is without effect on the excretion of calcium by the liver.

Three experiments involving intravenous injections of calcium chloride were carried out. Dog 26 was given three 10 cc. injections of a

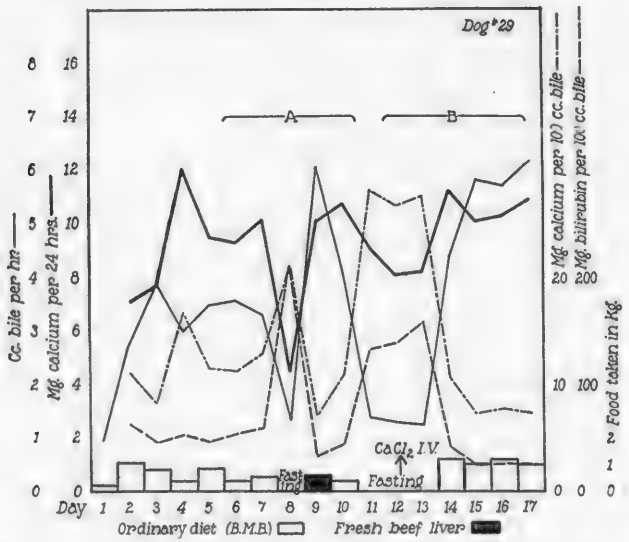
Dog 29
A
B
Cc. bile per hr.
Mg. calcium per 24 hrs.
Mg. calcium per 100 cc. bile
Mg. bilirubin per 100 cc. bile
Gm. food eaten in Kg.
CaCl2 I.V.
Fasting
Day 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Ordinary diet (B.M.B.)
Fresh beef liver

CHART 7. The effects of fasting, liver feeding, and the intravenous injection of a calcium salt on the character of the bile. On the 12th day three 10 cc. intravenous injections each of 10 per cent calcium chloride were given.

10 per cent solution of $CaCl_2$ in water at 2 hour intervals on the 19th day of intubation. Dog 29 received the same amounts on the 12th day, and Dog 16 two such injections at an interval of 5 hours on the 56th day. The results as concerns the bile are recorded in Charts 6, *B*, 7,*B*, and 4,*B*. Introduction of even these large amounts of calcium into the blood stream had no demonstrable effect to increase the excretion of the element by the liver. The slight variations to be observed in the charts are well within the ordinary range.

The Gall Bladder and the Bile Calcium.

There has been much debate as to the influence of the gall bladder on the calcium content of the bile. In a series of animals in which the common duct was obstructed by severing it between ligatures, the calcium content of the stasis bile found in the gall bladder was compared with that of the original contents of the organ as procured through a catheter run up through the common duct at the initial

TABLE I.

No.	Days of obstruction.	Character of bladder contents.	Bilirubin per cc. bile.	Ca per 100 cc. bile.	CO_2 per liter bile.*
			mg.	*mg.*	*gm.*
1	0	Watery, clear, yellow-brown.	0.47	22.8	1.76
	3	Greenish black.	8.42	59.0	0.82
2	0	Pale amber, clear.	0.56		1.37
	5	Brownish black.	4.66	53.3	0.13
3	0	Amber, clear.	0.42	38.0	0.63
	6	Greenish black.	2.2	44.6	0.50
4	0	Dark brown, flaky.	1.03	28.5	0.79
	4	" "	1.78	21.8	1.57
5	0	Pale yellow.	0.3	30.7	
	2	Greenish black.	4.26	47.4	
6	0	Pale yellow.	0.18	30.7	
	2	Greenish black.	9.61	53.0	

*The CO_2 was determined by the method of Van Slyke (Van Slyke, D.D., *Proc. Nat. Acad. Sc.*, 1921, vii, 229). The figures in this column indicate that the gall bladder is able to reabsorb this compound to a remarkable degree, as in the case of No. 2.

operation. The operative procedure has already been described.[3] The calcium, bilirubin, and carbonate findings are given in Table I.

The enormous pigment concentration within the gall bladder occurring in some of these instances is attributable in the main to the functional activity of the gall bladder to concentrate the bile,[7] though certain other factors discussed in another paper[3] may have been responsible in minor degree. The calcium content of the stasis bile in

contrast to that of bilirubin was never very great. Indeed, the concentration per cc. was well within the normal range for animals taking little food. While there was usually some increase over the concentration in the specimen obtained at operation, in only one instance was there so much as a doubling, and that the one in which the bilirubin underwent a 58-fold increase. If the bile elaborated immediately after operation held the usual amount of calcium—and all the evidence from the fistula animals of the present work would go to show that this is the case—then one can only account for the failure to find an abundance of the element in the inspissated stasis bile by assuming that its salts passed out through the wall of the gall bladder. Hoppe-Seyler[12] has shown that sodium and potassium do so pass out and Brand[13] that the freezing point of gall bladder bile, no matter how thick, is the same as that of liver bile, a relation maintained through the removal of salts and water from the organ by the processes of osmosis and diffusion. A considerable portion of the calcium of dog bile is present in the form of carbonate[1] which should lend itself well to such elimination. That most of the calcium present in bile exists in the form of dialyzable compounds is evident from my findings.

The Calcium Content of "White Bile."

In a previous paper from this laboratory[14] the fact has been brought out that the bile ducts do not, like the gall bladder, act to concentrate the bile but instead tend to dilute it with a secretion of their own which is colorless and devoid of bile salts and bilirubin, even when the animal is deeply jaundiced. The "white bile" found above an obstruction in a duct system cut off from the ordinary connection with the gall bladder consists of such pent-up secretion. The calcium content of this fluid has considerable interest. For the purpose of obtaining specimens the common ducts and cystic ducts of three normal dogs were cut between ligatures. The operations were performed aseptically, under ether. Between 2 and 3 weeks later the animals were

[12] Hoppe-Seyler, F., Physiologische Chemie, Berlin, 1878, pt. 2, 302.
[13] Brand, J., *Arch. ges. Physiol.*, 1902, xc, 491.
[14] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 75.

chloroformed and the contents of the dilated ducts were removed, cultured in bouillon and on agar, and analyzed for calcium. The results are presented in Table II.

It will be seen from the table that the calcium content of the "white bile" was at the lower limit of the range encountered in ordinary fistula biles.

Under the circumstances of progressive obstruction to the outflow of bile from the liver, the amount of the secretion formed and its content in bilirubin both progressively fall off.[15] In Dog 18, Chart 1,*A*, such obstruction developed through a kink in the tubing, which was later relieved. The findings are anomalous in that the bilirubin concentration per cc. rose as the obstruction increased instead of falling as happens usually. After the obstruction was relieved the bile had a very low pigment content, and low calcium concentration.

TABLE II.

Dog No.	Period of obstruction.	Character of duct contents.	Results of cultures.	Calcium content per 100 cc.	Range, under ordinary conditions, of the calcium contents of the bile of six fistula animals.
	days			*mg.*	
37	19	Light yellow.	Sterile.	10.6	7—52 mg. per 100 cc. of bile.
22	14	White tinged with yellow.	"	10.0	
25	17	Light yellow, watery.	"	7.7	

In another animal in which only the bile from approximately one-third of the liver was collected—by intubation of the duct from this portion—while the remainder of the secretion reached the intestine by way of the ordinary channels, there was transient and incomplete obstruction in the collecting system. The bile changes are recorded in Table III.

It will be seen that whereas the bile quantity and the bilirubin concentration had fallen off greatly when the obstruction was at its height, the calcium concentration had diminished to but a negligible degree. It was still within the lower limit of the range that is "normal" to fistula animals. The total output of calcium, on the other hand, was greatly lessened, by corollary, to the lessening of the bile amount.

[15] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 685.

"White bile" is met with under yet another set of experimental conditions. It has long been known that animals given toluylenediamine[16] or phosphorus[17] may at the height of liver injury yield only a glairy, approximately colorless fluid.

This change in output lasts but a few hours as a rule and is usually preceded and followed by the elaboration of a heavily pigmented, thick secretion. Chloroform poisoning, on the other hand, as recent observations in this laboratory attest, results in a progressive and sometimes practically complete suppression of bile with, as the fluid output lessens, a marked diminution in bilirubin such that the final cubic centimeters of secretion are practically colorless. This ultimate

TABLE III.

Days after intubation.	Appearance of the bile.	Calcium per 100 cc.	Bile per hr.	Bilirubin per cc.
		mg.	*cc.*	*mg.*
2	Dark brown, clear, watery.	12.7	2.39	0.555
3	" " " "	13.6	2.49	0.521
7	Very light brown, clear, watery.	12.7	0.42	0.229
8	" " green, " "	11.4	1.21	0.114
9	" " " " very watery.	11.5	1.48	0.092
10	" " brown, " " "	12.9	3.09	0.113
11	Light brown, clear, watery.	12.6	2.81	0.135
12	" " " "	12.9	3.33	0.181
13	" " " "	13.5	3.17	0.267
14	" " " "	12.9	2.75	0.301

fluid is thin and resembles in general character the "white bile" which assembles above an obstruction. The animal may remain in good condition throughout the changes which endure only 48 to 72 hours at most, and are rapidly recovered from; but in instances in which bile suppression is complete, death is more usual. The findings in dogs in which the suppression was not quite complete are illustrated in the charts. It will be seen that in one instance (Chart 2,*B*) the calcium concentration of the bile remained practically stationary throughout the disturbance, whereas in the other (Chart 5, Section

[16] Afanassiew, M., *Z. klin. Med.*, 1883, vi, 281.

[17] Pilzecker, A., *Z. physiol. Chem.*, 1904, xli, 157.

B), in which recovery was complicated by a supervening obstruction within the tube system, the calcium concentration rose somewhat during the period when the suppression was at its greatest.

The evidence at hand does not permit of the conclusion that the "white bile" secreted after liver injury and that formed on progressive obstruction to outflow are identical in source with the "white bile" of frank obstruction; that is to say, are duct products. But the fact that they tend to be weak not only in pigment and salts but also in calcium may be not without significance in this relation.

DISCUSSION.

In the animals of the present study the calcium of the fistula bile varied under normal conditions from 0.0061 to 0.0578 per cent. Hoppe-Seyler found 0.031 per cent calcium in his single instance and Jankau's figures vary between 0.013 and 0.017 per cent. These workers had noted that the average percentage under normal conditions is approximately the same as that in mucous secretions of other derivation and they supposed the calcium not to be secreted by the liver like the bile constituents proper but by the lining of the bile passages. They made no observation on the variations in calcium output under physiological conditions. These variations as described in the present paper leave no doubt that most of the calcium must be eliminated through the liver, unless indeed one is willing to suppose that the mucous membrane of the biliary passages varies in its activity to put out calcium synchronously with the activity of the liver tissue proper to put forth bile. The calcium concentration of that "white bile" which is an undubitable secretion of the bile passages is always low as is the volume output. But when the bile secretion itself is much diminished as the result of an ordinary physiological influence, fasting, the calcium concentration is frequently great.

That the amount of calcium in the duct secretion proper should be so small is a point worth emphasizing because many writers have referred the calcium of cholelithiasis to this source. Needless to say the present findings throw no light on the content of calcium in the secretion of inflamed duct systems.

Jankau[5] reached the conclusion from observations involving an intermittent and far from complete collection of bile that calcium feeding does not increase the output of the element. The present findings with the total bile prove this to be the fact. Jankau fed his animals diets rich in calcium and assumed that it would be absorbed from the intestine, since previous workers[18] had found an increase in the urine after administration of calcium salts by mouth to normal dogs. In view of later work it is questionable whether an animal with a bile fistula can absorb much calcium from the food. Telfer[19] noted that in infants in which there was a lack of bile in the intestine, owing to congenital obliteration of the ducts or syphilitic hepatitis, the calcium given in the diet was very largely combined with fatty acids to form soaps in the intestine and appeared as such in the stools. Other workers[20] have described softening of the bones in animals with long-standing biliary or pancreatic fistulas,—a fact which would suggest a gradual depletion of the body content of the element. This depletion can scarcely be ascribed to the small amount of calcium leaving the body by way of the liver, for far more is excreted normally by way of the large intestine. Rather may one suppose that lack of bile or pancreatic juice in the intestine results in a lessened calcium absorption therefrom. But a metabolic disturbance within the organism cannot be ruled from possibility as the explanation of the bony changes.

In some of the experiments of the present work, uncertainties as to calcium absorption from the intestinal tract were avoided by administering calcium salts intravenously. Rey[21] claims that the blood calcium remains elevated for more than 4 days after an intravenous injection of 0.8 gm. of oxide of lime. Salvesen, Hastings, and McIntosh[22] find, however, that after the injection of 0.5 to 1 gm. of calcium, as calcium chloride, the calcium concentration in the blood is elevated

[18] Riesell, A., in Hoppe-Seyler, F., Medicinisch-chemische Untersuchungen, Berlin, 1866–71, 319. Soborow, S., *Centr. med. Wissensch.*, 1872, x, 609.

[19] Telfer, S. V., *Biochem. J.*, 1921, xv, 347.

[20] Babkin, B. P., *Zentr. ges. Physiol. u. Path. Stoffwechs.*, 1910, v, N. S., 561. Whipple, G. H., *Physiol. Rev.*, 1922, ii, 440.

[21] Rey, J. G., *Arch. exp. Path. u. Pharmakol.*, 1894–95, xxxv, 295.

[22] Salvesen, H. A., Hastings, A. B., and McIntosh, J. F., *J. Biol. Chem.*, 1924, lx, 327.

for not more than 6 hours and in one case it was so for but 3 hours. They also find no increase in the calcium content of the urine or feces during the 6 days after intravenous administration, and conclude that the element must be stored somewhere in the body. Their observations on the blood will go far to explain the absence of any increase in the calcium output in the bile after intravenous injections, as in the experiments described in this paper, of as much as 0.25 gm. per kilo of calcium chloride—more than three times the greatest amount employed by Salvesen and his associates.

There is much diversity of opinion as to the source of the calcium of gall stones.[23] Many have supposed that the mucous glands of the gall bladder add calcium salts in quantity to the bile. Dochmann[24] compared the calcium and sodium present in the liver and bladder biles of the same dog and found the following relative amounts per 100 gm. dried substance.

	Ca	*Na*
Liver	7	13
Bladder	24	10

He infers from these figures that the gall bladder adds calcium to its contents. The stasis experiments of the present work show clearly that calcium is resorbed by the normal gall bladder, not secreted by it. Dochmann's figures are readily explained on the assumption that sodium salts pass out through the gall bladder wall with far greater ease than do those of calcium. Calcium would seem to hold a position, in this respect, intermediate between bilirubin, which is presumably not resorbed at all, and the sodium salts.

The present work does not suffice to answer the question whether, under pathological conditions, there are sources of calcium over and above the normal—as calcium in the exudates of inflamed bile passages or from abnormally overstimulated mucous glands. It does, however, provide some few facts toward an ultimate understanding of the circumstances under which calcium stones may be expected to form. For example, the element tends to be present in the bile in great amount during fasting. Other things being equal, one might

[23] Lichtwitz, L., *Ergebn. inn. Med. u. Kinderheilk.*, 1914, xiii, 1.
[24] Dochmann, A. M., *Wien. med. Presse*, 1891, xxxii, 1198.

suppose this physiological state to predispose to stone formation. However, there is to be noted during a fast, concomitantly with the increase in calcium, a change in the reaction of the bile,[3] such as would tend to prevent the falling out of calcium salts. It is conceivable that, under circumstances of gall bladder derangement, the change may not be brought about and stones may occur in consequence. But there are so many other possible factors to be considered, owing to the complexity of the bile and of the influences of the channels with which it comes in contact, that this can be no more than a suggestion.

It is of interest to contrast the behavior of calcium with that of another constituent of bile, cholesterol, which is of extreme importance in relation to gall stones. Cholesterol, like calcium, becomes highly concentrated in the bile when the volume output is small,[25] as during fasting. But cholesterol, in contrast to calcium, is greatly increased both in concentration and in total amount, by the administration of large amounts of it in the food. Whereas "white bile" always contains an appreciable amount of calcium, its cholesterol content is almost nil, indicating that there is but one important source of the latter—the liver cells—whereas calcium is secreted both by the liver cells and by the lining of the bile passages.

SUMMARY.

A day to day study has been made of the calcium content of the total liver bile of dogs intubated under sterile conditions. The concentration of this element in the bile is fairly constant under physiological conditions which do not involve wide fluctuations in the secretory output. It follows that the calcium yield for each 24 hour period in general varies directly with the amount of the bile. But when this amount becomes greatly lessened, as the result of fasting, the concentration of calcium becomes markedly increased, though not sufficiently so to compensate for the lessened volume. When the bile amount rises much above the average after the ingestion of food in quantity, the calcium content, unlike that of pigment, does not become correspondingly diminished, but tends to remain the same as ordinarily. Hence when great amounts of bile are put out, so too are rela-

[25] McMaster, P. D., *J. Exp. Med.*, 1924, xl, 25.

tively great amounts of calcium. This is not because of the increased ingestion of the element. For neither feedings with bone meal, nor the administration of large quantities of calcium salts intravenously or by mouth has any effect to alter the biliary output of calcium.

The normal gall bladder, far from secreting calcium into the bile, as some have supposed, acts to remove this element from the secretion, and removes carbon dioxide as well.

The "white bile," which is a specific secretion of the bile ducts, contains but little calcium, like the mucous secretions from elsewhere in the body. The concentration is only slightly greater than that in the blood plasma, and contrasts significantly with the high concentration to be noted in true bile of the fasting animal. Evidently the greater portion of the bile calcium must be secreted, not by the duct walls, but by the liver itself.

The findings have an evident bearing on the problem of cholelithiasis.

STUDIES UPON THE ETIOLOGY OF DENGUE FEVER.

I. Experimental Transmission to the Lower Animal.*

By WILLIAM H. HARRIS, M.D., and CHARLES W. DUVAL, M.D.

(*From the Departments of Pathology of Tulane University of Louisiana and the Charity Hospital, New Orleans.*)

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The epidemic of dengue fever which occurred in certain of the Southern States during the fall of 1922 and the winter of 1922–23 afforded us the opportunity for investigating the nature of the virus. The disease has received little attention from the standpoint of research since the human transmission experiments of Ashburn and Craig[1] demonstrated the filterability of the virus and its existence in the circulating blood of man. Inasmuch as the disease has appeared at relatively infrequent intervals, the last outbreak in this country occurring in 1907, it was considered that added information concerning the nature of the virus could be obtained with the newer methods of investigation which have been introduced since that time.

The attempts at transmission of dengue fever to the lower animal have on the whole proven unsuccessful. It is probable that in the earlier experiments no effort was directed toward ascertaining the febrile and leucocytic response induced in the inoculated animal. For this reason it is likely that positive reactionary phenomena were overlooked.

Yamaguchi, Ditsumi, and Tonomura[2] report fatal infection in guinea pigs

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[1] Ashburn, P. M., and Craig, C. F., *Philippine J. Sc.*, 1907, ii, 93.

[2] Yamaguchi, K., Ditsumi, D., and Tonomura, K., reviewed in *China Med. J.*, 1916, xxx (6), 460, cited by Chandler and Rice.[3]

[3] Chandler, A. C., and Rice, L., *Am. J. Trop. Med.*, 1923, iii, 233.

inoculated with blood from human dengue fever; however, they were unable to transmit the virus beyond the third generation. Chandler and Rice[3] report negative results in the guinea pig inoculated with human dengue blood, though they mention the occurrence of a papular eruption in a *Macacus rhesus* monkey, which appeared 5 days after inoculation. These authors state that they did not regard this rash as significant of transmission of the disease.

For the purpose of transmitting dengue fever to the lower animal or of producing a reaction indicative of infection, experiments with the guinea pig, rabbit, and monkey were carried out by inoculating these animals with the blood from selected human cases of the disease. In view of the success of Noguchi[4] with yellow fever, and that of one of us (Duval[5]) with the guinea pig in the study of measles, this species was extensively used throughout the study, and as early in the work it was found to be the animal of choice, it was soon used exclusively in the series of experiments herein reported.

The human dengue material employed was blood freshly drawn by venipuncture from patients presenting typical symptoms of the disease, care being used to select only those with temperature, leucopenia, and exanthem. From such cases the blood was secured on the 2nd or 3rd day of the primary pyrexia, which usually is the time of the appearance of eruption. Immediately after withdrawal the blood was defibrinated or citrated and a portion injected intraperitoneally or intracardially into the experimental animals. Another portion of the defibrinated blood was centrifugalized and the serum collected and passed through a Berkefeld filter (N), the filtrate being used for inoculation. With the material from the human cases, this plan of employing whole defibrinated blood and Berkefeld filtered serum for animal inoculation was followed. The amount of material used for injection was usually 0.5 to 1.0 mil of defibrinated blood and 0.25 to 0.5 of filtered serum.

Simultaneously with the inoculation of animals, cultures from human dengue material before and after Berkefeld filtration were prepared upon a variety of media to determine the possible presence of cultivable microorganisms in the circulating blood. Furthermore, a thorough dark-field study was made of all materials used.

[4] Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547.

[5] Duval, C. W., and D'Aunoy, R., *J. Exp. Med.*, 1922, xxxv, 257.

The animals employed for each human case were carefully studied with respect to circulating leucocytes and pyrexia before inoculation. Only animals with normal rectal temperatures (101–103°F.) and leucocytes (12,000 to 15,000 per c.mm.) were selected. By preference half grown guinea pigs (250 gm.) were employed in direct transmission experiments from the human case. The number of animals used for first passage of virus from each human case varied from four to eight, and often from twelve to sixteen. All inoculated animals were observed daily with respect to pyrexia and other signs of reaction; the leucocytes were usually counted every 2nd or 3rd day, and the temperature taken by rectum once or twice daily. It should be said here that the manner of taking temperatures and collecting blood for leucocytic counts has an important bearing upon the proper interpretation of the reaction. When inserting the thermometer it should be introduced into the rectum as far as the normal marking, otherwise an irregular and inaccurate reading is obtained which is often 1–2° lower than the true rectal temperature of the animal. Blood for leucocytic determinations is collected from the marginal ear vein of the animal by pricking with a sterile needle, care being exercised not to use the same site for subsequent collections. These features may appear of minor importance, but as the experimental reaction consists in pyrexial rise and leucocytic fall, it is essential to follow a standard method.

Certain of the inoculated animals which for several days showed a rise of temperature above 104°F. or a marked fall in the circulating leucocytes were killed, and transfer of the blood and serum filtrate made to other animals of the same species. Reacting animals that had recovered were kept under observation and subsequently used for determining the degree and duration of the immunity conferred.

EXPERIMENTAL.

For the sixteen human cases of dengue fever studied by us, 143 guinea pigs were used in the initial attempt at inducing infection. Out of this number 42 animals, representing eleven human cases, developed a characteristic pyrexial and leucocytic reaction following an incubation period of from 3 to 5 days. The reaction, in as far as temperature and leucocytes are concerned, was identical with that

observed in human dengue fever. The rise in temperature, generally sudden, was often maintained at a height of 2–4° above the normal for a period of from 3 to 4 days, after which it fell to normal. In over 50 per cent of the reacting animals of the initial series, there occurred a secondary rise in temperature after an interval of 24 to 48 hours; this rise did not as a rule attain the height of, or last as long as the primary pyrexia. It is of interest here to note that remission and subsequent elevation of temperature are not infrequent for the human case ("saddleback" curve). Some of the inoculated animals which did not develop temperature showed, however, a well marked leucopenia; infection must of necessity have occurred in these pigs as they failed to react to subsequent injections of infective doses of virus blood.

After apparent recovery from the reaction induced by the inoculation of dengue material, the animals were normal as far as could be determined; *i.e.*, they were lively, feeding well, with hair slick and smooth. It was noted that subsequent to this period death occurred in from 3 to 5 weeks after the date of inoculation in a large percentage of the recovered animals. For several days prior to death the animals though apparently in good health became markedly emaciated and anemic. At autopsy no gross lesions were demonstrable; especially is it noteworthy that the spleen and lymph nodes were normal in most instances. The cause of death in some of these animals is not understood, while in others it is undoubtedly due to an intercurrent infection. In this connection cultures from the heart's blood of certain reacting animals show the presence of microorganisms of the colon-paratyphoid group. Since the virus serum was always filtered and tested culturally before using, it is evident that the secondary infection in the inoculated guinea pig did not come from contaminated injection material, but originated within the animal, presumably the intestinal tract.

The occasional presence of paratyphoid organisms in the blood of the inoculated guinea pig nevertheless obligates the consideration of their relationship to the reactionary phenomena described for experimental dengue. The possibility of transferring paratyphoid from one animal to another is, however, excluded by the employment only of culturally controlled filtered virus blood. The use of serum

filtrate free of any organism of the colon-paratyphoid group and the small percentage of instances in which such organisms are found in the blood of the reacting animal demonstrate that their occurrence is only accidental. While it is in order to mention the subsequent occurrence of the colon-paratyphoid organisms occasionally in the blood of certain animals injected with the virus of dengue, it is apparent that their rôle is one of secondary and terminal invasion.

Case 1.—Female, Italian, age 16 years, residing in Morgan City. Onset of illness Sept. 28, 1922. Ushered in with chill followed by intense headache, high fever, and severe pains over the body, more particularly the back and lower extremities. Sept. 29. Pains more general; made worse on motion of affected parts. Temperature 104°F.; patient shows photophobia. Sept. 30. Feeling better; pains not severe; temperature 103°F. Chest, arms, and face covered with a roseolar eruption. On this date 15 mils of blood were withdrawn from the median basilic vein, defibrinated, and a portion inoculated into guinea pigs at the bedside. Oct. 1. Patient was much better; temperature 101°F., leucocytic count 3,000, rash more marked. Oct. 2. Temperature normal, rash fading. From this time on patient made an uneventful recovery.

Animal Inoculation.—The citrated blood collected on the 3rd day of the disease was in part immediately injected intraperitoneally into six half grown guinea pigs, a dosage of 1 mil each being used; another portion of the blood was kept at room temperature for 24 hours, then centrifugalized, passed through a Berkefeld filter, and the filtrate injected intracardially into six guinea pigs.

2 mils of the filtered virus blood were equally distributed to four anaerobic tubes containing a mixture of Ringer's solution three parts, and rabbit serum one part, with 0.3 per cent neutral agar. The inoculated medium was incubated at 37°C. for a period of 17 days. All tubes remained sterile with respect to ordinary microorganisms. 1 mil of the culture mixture was injected intraperitoneally into four guinea pigs, each receiving 0.25 mil of the culture material.

Of the three different sets of pigs receiving virus blood, sixteen in all, five reacted; two that were inoculated with defibrinated blood, two that received filtrate, and one of the pigs injected with culture. As the reaction was similar in all of the reacting animals only the protocols of three, one of each series, are given.

Guinea Pig 2.—(Defibrinated blood series.) Sept. 30, 1922. Inoculated intraperitoneally with 1 mil of fresh defibrinated blood. At time of injection, temperature 102°F., leucocytes 14,000. 4 days after inoculation the temperature rose to 104.3°F., on the 5th day to 105°F., 6th day 104.2°F., dropped to 102°F., on the 7th day. The temperature rose again on the 8th day to 104.3°F., and on the 9th day returned to normal and remained so. The leucocytic count on the 1st day of pyrexia was 12,000; 2 days later, or on the 3rd day of fever, it had dropped to 7,500, and on the 8th day was down to 3,250. Oct. 14. The leucocytic count was 16,500, and the temperature 102.3°F. Animal normal in other respects.

Guinea Pig 5.—(Filtrate blood series.) Oct. 1, 1922. Inoculated intracardially with 0.25 mil of Berkefeld filtered virus blood. Temperature before injection 101.4°F., leucocytes 16,000. 5 days after inoculation the temperature was 105°F.; on the 6th day it dropped to 104°F., to rise again on the 7th day to 106°F. On the 8th day the temperature was 104.3°F., and the leucocytes had fallen to 7,500, a drop of over 50 per cent. The animal for the first time appeared ill, with hair ruffed, refusing food and remaining off in corner of cage. Oct. 8. Animal killed for examination and passage of virus.

The autopsy showed no gross changes in any of the organs except the spleen which was slightly enlarged. Cultures prepared from the heart's blood remained sterile 48 hours after incubation of 37°C. Blood was removed from the heart before death, defibrinated, centrifugalized, and filtered for passage to other guinea pigs.

Guinea Pig 4.—(Culture series.) Oct. 18, 1922. Inoculated intraperitoneally with 0.25 mil of 17 day old culture. Temperature 101.2°F., leucocytes 11,500 before injection. 3 days after inoculation the temperature rose to 105°F., on the 4th day it was 106°F., and on the 5th day 104.3°F. The animal became inactive and irritable, and handling gave rise to pain. On the 3rd day of the pyrexia the leucocytes dropped to 6,000. The animal recovered completely in 10 days.

Case 2.—White boy, age 11, native of plantation district in the outskirts of Morgan City. Patient was perfectly well prior to present illness. Onset Oct. 2, 1922. Began with chill, headache, photophobia, and cramp-like pains in arms and legs. Temperature on Oct. 3, 103°F., leucocytes 4,500. Patient complained of aches and pains over the body, more severe in back of neck. Fine macular eruption, most marked on legs and arms appeared Oct. 4, 3 days after the onset of illness. Oct. 5. The temperature was normal and the rash was fading. No secondary rise in temperature; patient made rapid recovery and was perfectly well Oct. 9. Blood was obtained for study on the 2nd day of illness.

Animal Inoculation.—Six guinea pigs were injected Oct. 4, 1922, intracardially, with 2 mils of defibrinated blood which had been kept overnight at room temperature. Cultures prepared from a portion of the blood upon nutrient media remained sterile after 3 days incubation at 37°C. Dark-field study was negative. Two of the six pigs inoculated reacted. In one the temperature suddenly rose on the 4th day to 105°F., the 5th day it was still 105°F., and on the 6th day 104°F. The leucocytes had fallen from 12,500 to 4,000. The animal did not appear sick; hair slick and feeding well. Oct. 9. The pig was anesthetized and 6 mils of blood withdrawn from the heart for examination and further passage. Bleeding did not kill the animal, which was lively and apparently well the following day; however, the temperature was 106°F. Oct. 11. The temperature had dropped to 103.2°F., from which date it remained normal. Blood withdrawn from the heart at the time of highest temperature was injected into four passage animals of the same species. No temperature or leucocytic reaction was evinced in any of these though observed for a period of 3 weeks. The second reacting pig of this case developed pyrexia of 104.4°F. on the 3rd day following the inoculation. On the 4th day it was 104.3°

F., and dropped within the normal range the next day, to rise again to 104°F. the succeeding day; leucocytes were 10,500. The second temperature rise lasted only 24 hours. The animal was killed 48 hours after the subsidence of fever.

Autopsy.—All organs appeared normal except the mesenteric lymph nodes and spleen which were distinctly enlarged. The spleen was smooth, tense, firm in consistence, and of a dark red color. On section the cut surface appeared comparatively dry and the markings well defined. The mesentery lymph nodes were the size of peas and of a uniform pale yellowish white color; no hemorrhagic areas were detected.

Microscopic examination of spleen and lymph nodes showed foci, particularly in and about the Malpighian bodies and germinal centers, of endothelial proliferation. Cellular elements of an acute inflammatory character were absent. Cultures prepared from the heart's blood were sterile.

Attempts to induce reaction in four passage animals with the defibrinated blood from this guinea pig were unsuccessful. Failure to perpetuate the virus of this second positive transmission is difficult to explain in the case of the pig whose blood was collected at the time of highest temperature; however, in the second reacting animal, negative passage experiments were most likely due to the fact that the blood was withdrawn 2 days after subsidence of the fever and presumably did not any longer contain adequate virus.

Case 3.—White man, age 26 years, taken sick suddenly Nov. 5, 1922, complaining of burning sensation in eyes and pains in the back, arms, and legs; temperature 102°F. 2 days after onset a macular rash appeared on forehead, chest, and arms. On this date patient was greatly prostrated and visibly sick. Leucocytic count 3,250. 20 mils of blood were removed from the median basilic vein of patient. 5 mils were placed in 2 per cent citrate and 15 mils were defibrinated and centrifugalized. The supernatant serum was passed through a Berkefeld filter and the filtrate used for cultural study and animal inoculation. Cultures of the blood upon the ordinary media made at time of bleeding remained sterile. Microscopic examination of blood smears stained by Giemsa and Wright methods revealed nothing of special note. Dark-field study of the citrated and defibrinated blood showed nothing of interest. Ten tubes of Noguchi's media for the cultivation of protozoa anaerobically were inoculated as follows: four tubes received 0.5 mil each of filtered serum from the case, two tubes 0.75 mil each of unfiltered serum, and four tubes 0.5 mil each of the defibrinated blood.

Animal Inoculation.—Twelve guinea pigs were employed; four received the filtered serum in dosage of 1 mil each, four the defibrinated blood, and four received 1 mil of culture mixture 8 days after its incubation at 37°C. Out of the three sets of animals, six reacted; one with the serum filtrate, two with the whole blood, and three with the culture. All reacting animals showed symptoms not earlier than the 3rd day or later than the 6th day. In two animals the duration of the fever was 2 days, dropping suddenly thereafter and remaining normal. In three pigs there was a primary pyrexia of 105–106°F. for 2 to 3 days, then a return to normal for a period of 36 to 48 hours, with a secondary rise of 1–2° which lasted

from 1 to 3 days. The leucocytic counts were in all reacting pigs below the original, and in two animals as low as 5,250 and 3,000 respectively.

Autopsies.—Pig 2 of the culture set, Pig 4 of the defibrinated blood set, and Pig 1 of the filtrate set, were killed at the time the temperature reaction was at its height, and the following observations made. Cultures from the heart's blood were negative for known cultivable microorganisms. No gross changes were noted in two of the animals. In the pig which received serum filtrate the spleen was approximately three times its normal size, firm, smooth, and dark red in color. On section the Malpighian bodies were distinctly visible as grayish white elevated dots. The lymph nodes in the mediastinal and intraperitoneal regions were moderately enlarged. Microscopically the lesions showed a marked proliferation of the endothelium. There was no excess of neutrophils or other evidence of acute inflammation.

Virus Passage.—From reacting animals of this case filtered guinea pig blood was injected intraperitoneally into eight animals of the same species; two of the pigs of the second passage reacted in a characteristic manner. Filtered blood was transferred from one of these to a third passage set of eight pigs, three of which reacted with fever and leucopenia. The filtered blood from a typically reacting animal of the third passage was carried over into six pigs of a fourth passage, two of which reacted.

Case 4.—White male, age 25 years, native of New Orleans. Onset of illness Oct. 15, 1922. Began with headache, pains in back and arms, no respiratory symptoms; temperature 2nd day 103.2°F. Maculopapular eruption appeared on shoulders and arms, becoming more marked on Oct. 17; leucocytic count 4,000 on 3rd day of illness. 10 mils of blood were withdrawn from median basilic vein on Oct. 16, placed in citrate, and animals inoculated intraperitoneally within 1 hour after its removal.

Animal Inoculation.—Ten guinea pigs were used for the blood transfer. Five received intraperitoneally 1 mil each of citrated blood, and five were injected intracardially with a similar quantity of the whole blood. Of the inoculated animals only one reacted characteristically.

Guinea Pig 8.—At the time of inoculation the temperature was 101.2°F. and the leucocytes 14,750. 3 days after injection the temperature suddenly rose, remaining elevated for 3 days at 104.3°, 105.2°, and 106°F. respectively. On the day following, the temperature dropped from 106°F. to 102°F., to rise again the succeeding day to 105°F., returning to normal on the 8th day. Thus the "saddle-back" temperature curve was noted in this animal. During the febrile reaction the leucocytic counts were as follows: 12,500, 10,000, 8,500, and 6,250. The animal was killed for blood transfer on day following subsidence of the temperature reaction. For second animal passage of the virus, four guinea pigs were employed Oct. 26, 1922. Each received intraperitoneally 0.25 mil of filtered defibrinated blood from a reacting pig of first passage within 1 hour after its withdrawal. All four animals reacted after an incubation period of 4 days. The highest temperature was 107°F., and the lowest leucocytic count 2,000.

Autopsies.—Two of the second passage animals were sacrificed during the height of the temperature reaction for study and further passage. The post-mortem findings were negative except for enlarged spleen and lymph nodes. Cultures prepared from the heart's blood remained sterile.

Case 5.—Male, 32 years, native of New Orleans, enjoyed good health until time of attack. Onset Oct. 28, 1922. Headache and general malaise on the morning of the attack. In the afternoon of the same day patient was stricken on the street and was taken home in a dazed condition. His pulse was weak and he felt greatly prostrated; on the 2nd day of illness the temperature was 103.3°F. and patient complained of severe aches and pains over the body. Oct. 30. Blood was withdrawn from the arm vein for experimental study. 50 hours after the onset of the disease the temperature was 103°F. and the leucocytic count was 4,000. The following day the patient broke out with a typical rash which lasted 2 days. The "saddleback" temperature curve was noted in this case. Recovery was rapid after 10 days of illness.

Animal Inoculation.—A set of eight guinea pigs was inoculated intracardially with 2 mils of defibrinated blood within 3 hours after its withdrawal. Four animals of the series reacted with pyrexia and leucopenia; two on the 3rd day, one on the 4th day, and one on the 7th day following the inoculation. Three animals that failed to develop pyrexia showed around the 5th and 6th days a marked depression of the circulating leucocytes. The average temperature for reacting animals of this series was 105°F.

Animal Passage.—The virus blood from this case was carried through four passages; each succeeding transfer was made with filtered blood that was sterile with respect to ordinary microorganisms. Each passage resulted in more than 50 per cent of the animals reacting to inoculation.

Case 6.—Married woman, 38 years old, native of Italy, residing for past 10 years in New Orleans. Onset sudden with chill and fever on Nov. 11, 1922, followed by headache and severe pains in abdomen and legs. Patient was admitted to Charity Hospital Nov. 17, 6 days after the onset of the disease. At time of admission patient was slightly jaundiced, complaining of nausea and pains in the bones of the lower extremities. On this date the rash was fading, temperature 103°F., leucocytic count 4,000. Blood was removed from arm vein for experimentation Nov. 18.

Animal Inoculation.—Six guinea pigs were injected intraperitoneally with 2 mils of defibrinated blood within ½ hour after its withdrawal. Three animals of the series developed temperature and leucopenia after an incubation of 5 to 6 days, the reaction being in every respect similar to that of the animals of other cases. Second passage of the virus was not carried out.

Case 7.—White female, age 36, native of New Orleans. Illness began with chills and fever on Nov. 21, 1922, followed by pains in the back and severe headache. The temperature remained around 104°F. for 2 days, fell to normal on the 3rd day, to rise again to 101°F. on the day following. A roseolar eruption appeared during the secondary pyrexia and persisted for 48 hours. The leucocytic count

was 3,500. Blood was collected from the median basilic vein on the 2nd day of illness.

Animal Inoculation.—Each of six guinea pigs was injected intraperitoneally with 2 mils of defibrinated blood Nov. 22, 1922. Three of the animals reacted, two pyrexially and one leucocytically on the 4th day following the inoculation. The temperature in two of the reacting pigs persisted without much change for 4 days, when it fell to normal, to rise again after 48 hours remission, and remained elevated for 3 days ("saddleback" curve). The leucocytes in one of the animals were as low as 4,000 at the time of the secondary rise in temperature. In the other pigs little or no fall occurred for the circulating white blood cells. The filtered defibrinated blood from two of the reacting pigs was inoculated into four other animals of the same species. None of these showed subsequently any rise in temperature though observed daily for a period of 18 days. The leucocytic count in one of these pigs showed a fall from 11,000 to 8,000. In the other animals the white cells fluctuated within the normal limits during the entire period of observation.

Case 8.—Female, age 18 years, native of New Orleans. Onset of illness Nov. 21, 1922. Fever on 1st day of disease was 104°F. Patient complained of severe headache, pains in the back and extremities. On Nov. 24 a characteristic rash appeared on the face and chest and faded rapidly 2 days later. There was no marked remission of the fever during the 8 days of illness. Patient was bled on the 4th day of the disease and 12 mils of blood removed.

Animal Inoculation.—Six pigs were injected with the serum filtrate and four with whole blood, each receiving 0.5 mil intraperitoneally. Five animals of the set reacted typically in as far as temperature and leucocytes are concerned. All reacting animals showed the first rise in temperature on the 3rd day following the inoculation.

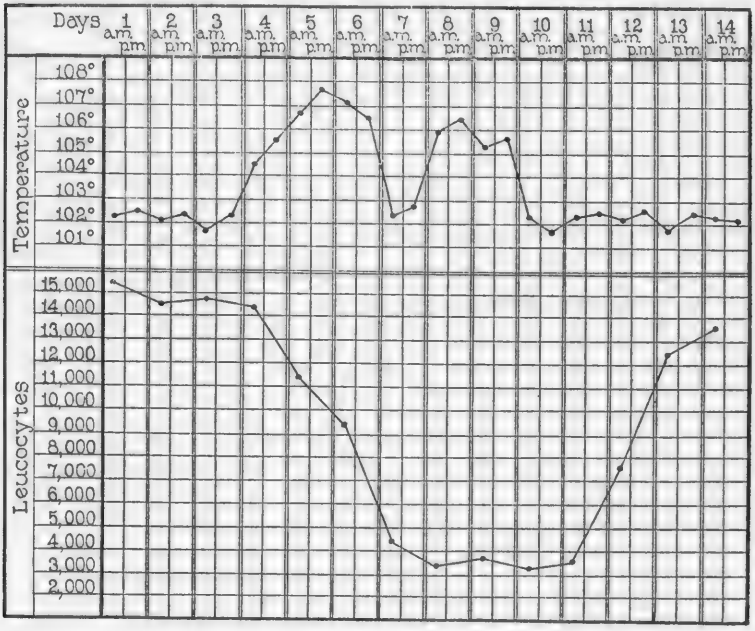
Guinea Pig 3.—The temperature was 104.3°F. on the 3rd day, 104.4°F. on the 4th day, 103°F. on the 5th day, 105°F. on the 6th day, 104.2°F. on the 7th day. In the course of the infection the leucocytes fell from 17,500 to 6,000. Animal died 10 days after inoculation. At autopsy the lungs revealed gross evidence of of lobular pneumonia. Cultures from the heart's blood showed *B. mucosus capsulatus* and bacilli of the colon-paratyphoid group. It is interesting to note that the sterile filtered blood from this animal produced in transfer pigs the typical reaction of experimental dengue fever.

Guinea Pig 6.—This animal reacted on the 3rd day following inoculation. The temperature for a period of 14 days was as follows: 102.2°F., 102.4°, 103°, 105°, 105°, 104.3°, 104.4°, 103°, 101°, 104.4°, 105°, 104°, 102°, 103.1°. Thus the so called "saddleback" temperature curve was depicted by the reaction of this animal—3 days incubation, 4 successive days of continued primary fever, and interval of 2 days remission of fever, followed by a secondary rise lasting for 3 days.

Passage Experiment.—The virus from Case 8 has been successfully propagated through 36 generations in the guinea pig. Out of 368 pigs employed for virus passage, positive reactions were obtained in 220. The proportion of "takes" has

steadily increased from the tenth passage, not infrequently every animal in a given series reacting.

Case 9.—Female, age 24 years, admitted to the Presbyterian Hospital, New Orleans, Nov. 29, 1922. The onset of the disease was ushered in on Nov. 27 with chills, headache, and pain in back and lower extremities. At the time of admission the conjunctivæ were injected, fever 103°F., and the body spotted with a macular rash. The leucocytic count was 6,250. 20 mils of blood were obtained on the 2nd day of the illness.



TEXT-FIG. 1. Leucopenia and pyrexial reaction ("saddleback" curve).

Animal Inoculation.—1.5 mils of citrated blood were injected into the peritoneal cavity of six normal guinea pigs. Two animals of the series reacted on the 5th day after inoculation. The reaction was identical to that observed in animals of other positive transmissions.

Case 10.—Male, age 56, Italian, living in New Orleans for past 10 years. Onset of illness Dec. 1, 1922. The disease began suddenly with high fever and chills; patient complained of headache, backache, and muscular soreness. The rash appeared Dec. 3, and was most marked on the arms and thighs. The temperature

ranged from 102–104°F. for 5 days, after which it fell to normal. 1 week later the temperature rose again and patient showed signs of broncho pneumonia from which, however, he recovered. Blood was obtained for study on Dec. 10. At this date the patient's temperature was 99.3°F., and the leucocytes were 7,250.

Animal Inoculation.—Seven guinea pigs were injected intraperitoneally each with 1 mil of filtered blood. Two animals reacted, one on the 3rd and one on the 5th day following the inoculation. Second passsage of the virus was not successful.

Case *11.*—Married woman, aged 23 years, 4 months pregnant, native of Louisiana. Onset of illness Dec. 8, 1922. Disease began with severe chill and fever (103°F.). On the 2nd day of illness a macular eruption appeared on the chest, arms, and thighs. The patient was prostrated and complained of headache and backache. Blood was withdrawn from the arm vein Dec. 10. At this date the temperature was 102.4°F. and the leucocytes were 3,000. The temperature persisted for 4 days, when it dropped to, and remained normal. Patient made uneventful recovery.

Animal Inoculation.—Sixteen guinea pigs were employed in the series, four receiving intraperitoneally 1 mil quantities of defibrinated blood, four serum filtrate, and four citrated blood which had been incubated at 37°C. for 14 hours. The several sets of animals were kept in separate cages along with an equal number of controls. All animals used in the experiment were normal at time of inoculation, temperatures below 103°F. and leucocytes between 10,000 and 16,000.

In addition to the human blood used for animal inoculation, a portion was diluted with sterile normal saline, centrifugalized, and the supernatant cell-free fluid passed through a Berkefeld filter. The filtrate was cultured for sterility and 1 mil quantities were used to inoculate tubes of Noguchi's leptospira medium. These were incubated at 37°C. for 14 days, after which time 1 mil quantities were transferred to sterile tubes of the same medium. These were likewise incubated for 2 weeks before executing a third subtransplantation. At the time there was no visible growth in any of the inoculated culture tubes.

Four guinea pigs were injected intraperitoneally with 1 mil quantities of the third generation culture. Three of the animals reacted in a characteristic manner.

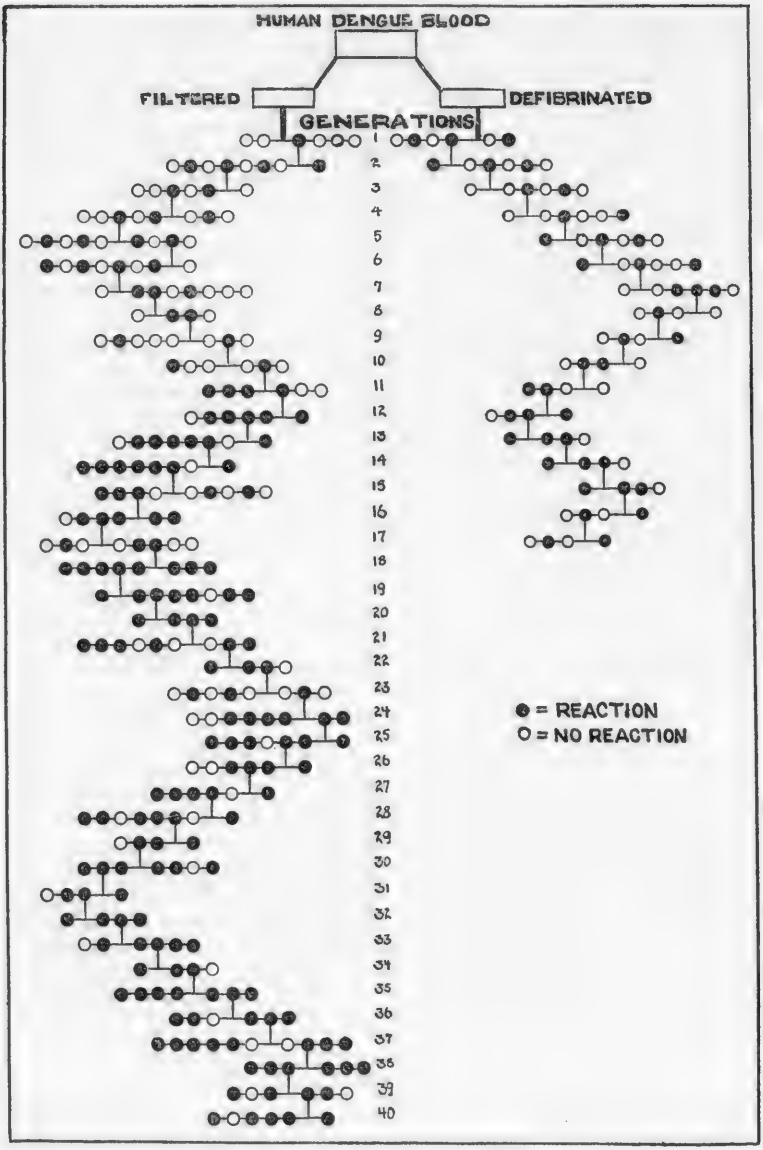
Of the four series of animals inoculated with materials from Case 11, nine reacted with respect to pyrexia and leucocytosis; one pig of the serum filtrate series, three of the whole blood, two of the citrated blood, and three that had received culture. Since the reaction was similar for all animals, the protocol of one only will be given.

Guinea Pig 4.—(Culture set.) The temperature rose to 105°F. on the 4th day following inoculation, was 104.3°F. on the 5th day, and on the 6th day dropped to normal. Fever again appeared on the 9th day and returned to normal on the 11th day. The leucocytes at time of inoculation were 12,000, 4 days later they were 3,000, and on the date of secondary rise in temperature they were 4,250.

Autopsy.—The animal was killed for study and blood transfer. At autopsy all the organs appeared normal except the spleen, lymph nodes, and liver, which were enlarged, especially the spleen. This organ was fully four times the normal size, of firm consistence, and of a dark red color. Microscopic sections show a marked

TABLE I.

Case No.	Sex.	Age.	Symptomatology.	Day of disease on which blood was collected.	Blood cultured at collection.	No. of pigs injected in primary series.	Character of inoculating material.	No. of reacting animals in primary series.	Subsequent passage generations.	No. of pigs injected in subsequent passage series.	No. of pigs reacting in subsequent passage series.
		yrs.									
1	F.	16	Chill; primary and secondary febrile stages; rash; leucopenia.	2	Negative.	16	Whole blood.	5	6	38	18
12	M.	23	Primary and secondary febrile stages; no rash; leucopenia.	2	"	8	Defibrinated "	0	0	0	0
2	F.	11	Chill; high temperature; rash; leucopenia.	1	"	6	Filtered "	2	4	10	7
6	"	39	Typical.	3	"	6	Citrated "	3	0	0	0
13			"	2	"	8	Defibrinated "	0	0	0	0
14	"	12	" rash lasted 4 days.	2	"	12	" "	0	0	0	0
4		25	" course; well marked "saddle-back" temperature; rash.	3	"	10	Citrated "	1	4	20	14
3			Typical; leucocytes 3,250 at time of collecting blood.	3	"	12	Filtered "	6	5	35	12
9			Typical; leucocytes 6,250 on 2nd day.	2	"	6	" "	2	6	48	22
5	M.	32	"	2	"	8	Defibrinated "	4	4	26	18
7	F.	22	" rash lasted 3 days.	2	"	6	" "	3	4	32	20
15	"	17	Mild case.	2	"	8	" "	0	0	0	0
10	M.	54	Typical course with subsequent relapse.	10	"	7	Citrated "	2	0	0	0
8	F.	18	Typical; 104°F. 3rd day; leucocytes 4,000 4th day.	4	"	6	Filtered "	5	35	260	
16	"	18	Typical case.	4	"	8	" "	0	0	0	0
11	"	26	Rash appearing at time of collecting blood. Temperature 103.4°F.; leucocytes 3,750.	4	"	16	Citrated filtered blood.	9	46	350	



TEXT-FIG. 2. Generation chart.

proliferation of the endothelium for the spleen and lymph nodes. The sections of the liver show extensive fatty and hydropic degeneration of the parenchyma.

Animal Passage.—The virus strain from Case 11 has been maintained through 45 consecutive animal transfers.

A confusing factor sometimes encountered in the experimentally reacting guinea pig to dengue virus is an intercurrent infection with one of the paratyphoid group of microorganisms. Not infrequently animals which have been inoculated with the uncontaminated filtered virus of dengue will develop during the course, or shortly following the subsidence of symptoms, a secondary infection which may obscure the dengue reaction. The secondary infection probably arises from the intestinal tract, from which bacteria invade the blood after the normal resistance has been lowered by the dengue virus. Occasionally these invasions occur in guinea pigs that at the time of inoculation with filtered virus were in all respects normal.

In spite of all precautions certain normal animals, whose blood was determined previously to be culturally sterile, would develop in a week to 10 days after inoculation with filtered dengue material a paratyphoid invasion of the blood. Experiments to determine the source of the secondary invader showed that it originates within the injected animal and most probably from the intestinal tract. Significant in this connection is the failure of controlled pigs to develop paratyphoid infection though kept in close contact with the inoculated animals throughout a 6 weeks period of observation. It is also noteworthy that guinea pigs fed with dengue virus failed to develop any reaction or a paratyphoid infection.

A sudden rise in the temperature of 2–3° in the dengue-inoculated pigs following a definite period of incubation, lasting, before returning to normal, for 2 to 3 days, and a concomitant fall in the circulating leucocytes we regard as the criteria in this animal of experimentally induced dengue. In those animals which become secondarily infected with paratyphoid, a low form of remittent fever continues for several weeks. Often the secondary infection is not recognized before several days after complete recovery from the effects of the dengue virus. The leucopenia likewise persists and often increases, while in the uncomplicated dengue pigs the white cells return to normal in a week to 10 days after the subsidence of the pyrexia.

There is no doubt that the paratyphoid bacilli will under accidental conditions give rise to a septicemia that may be characterized by a remittent type of fever and a leucopenia. This kind of infection undoubtedly accounts for much of the mortality in guinea pigs 6 to 8 weeks after complete recovery from the dengue inoculation.

Regarding the splenomegaly which is a fairly constant pathological feature in the guinea pig of experimental dengue, superimposed paratyphoid infection causes alterations in this organ of quite a different type. The paratyphoid splenic enlargement is due to an acute inflammation characterized by discernible tubercle-like discrete grayish white areas. On the other hand, the splenic tumor of the guinea pig of experimental dengue is proliferative in character and does not present macroscopic focal lesions.

SUMMARY.

The dengue cases studied during this epidemic were, as a whole, typical examples of the disease. The onset was sudden, often of a violent character, ushered in with severe headache and backache, vague pains throughout the body, and fever ranging from 101–105°F. On the 2nd or 3rd day a maculopapular eruption appeared, generally on the neck, chest, and arms, but sometimes more widely disseminated. In most instances the fever remained for 3 or 4 days, after which it dropped to normal to rise again in 24 to 48 hours. The secondary rise, while occasionally higher than the primary one, was as a rule of a milder character. A few cases occurred in which marked jaundice existed. As reported for previous epidemics, no fatalities were recorded and hence no human material for histological study was available. Those cases caught in the fastigium of the disease were selected when possible as a source of material. The leucocytic count in all of the observed human cases was below normal.

The experiments herein reported upon the transmission of dengue fever to the guinea pig are based upon the use of material secured from sixteen typical human cases of the disease, and upon the inoculation of many animals. Of the 143 animals used for the initial transmission, 42, representing eleven human cases out of the sixteen studied, reacted in a characteristic manner. The reaction occasioned in these animals by the inoculations closely resembles the symptoms seen in human dengue, differing only in the absence of exanthem.

The primary pyrexia following regularly after a definite incubation period of 2 to 5 days, the secondary rise in temperature after a 24 to 48 hour remission ("saddleback" curve), and the concomitant fall in the circulating leucocytes present a syndrome identical with that of the human disease.

Dark-field and special tinctorial studies of the dengue material, both human and experimental, have failed to reveal any visible spirochetal microorganism. It seems unlikely that an organism of any proportion other than a minute body is the excitant of dengue fever.

In the experimentally induced infection of the guinea pigs by the inoculation of dengue virus there occurs a rather constant and characteristic gross alteration of the lymphoid tissues, and of the spleen especially, which latter organ often attains a size three to four times that of the normal. Enlarged lymph glands in the peritoneal areas are also a fairly constant finding. The adrenal glands occasionally show marked enlargement. The histopathology consists of an endothelial proliferation occurring chiefly in connection with the Malpighian bodies of the spleen and the germinal centers of the lymph nodes.

Experiments performed with a view of demonstrating the protection afforded the recovered guinea pig were somewhat confused by delayed fatalities or later intercurrent infections to which the dengue-inoculated animal seems highly susceptible.

The constant and uniform results for all the positive cases indicate that the virus of dengue fever is not only transmissible to the guinea pig but is susceptible to propagation in this species through an undetermined number of successive generations.

After certain of the virus strains had been propagated through four or more generations they were discontinued. Two strains, however, have been continued for 36 and 45 generations respectively.

The equally successful results with Berkefeld filtered material and whole defibrinated blood indicate that the excitant of dengue fever belongs in the group of filterable viruses.

We wish to extend our thanks to Dr. Edmund Moss for his interest in procuring dengue cases, to Dr. Henry Daspit for valuable assistance, and to Dr. Charles de Gravelles of Morgan City for the opportunity of obtaining material from dengue patients in his locality.

STUDIES UPON THE ETIOLOGY OF DENGUE FEVER.

II. Cultivation and Nature of the Virus.

By CHARLES W. DUVAL, M.D., and WILLIAM H. HARRIS, M.D.

(*From the Department of Pathology of Tulane University of Louisiana, New Orleans.*)

Plates 36 to 38.

(Received for publication, July 5, 1924.)

While the exciting agent of dengue fever has been sought by almost every investigator who has studied the disease, few have attempted its cultivation by means of recent bacteriological methods. Ashburn and Craig,[1] though failing to cultivate the virus, demonstrated its filterability. All attempts to grow in artificial culture the virus of dengue fever have been unsuccessful. At least the results of those claiming to have cultured the specific excitant lack confirmation.

Since the etiology of dengue fever is only imperfectly understood we attempted to propagate the virus *in vitro* simultaneously with our efforts to transmit the disease to the lower animal, by inducing in them a reaction which would be indicative of the infection.[2] Consequently a part of the freshly collected human blood was cultured in special nutrients as well as in the more common media, the latter for detecting the readily cultivable bacteria. We had in mind at the onset of the work the possibility of dengue fever being caused by some spirochetal microorganism. The method used was essentially that of Noguchi[3] in his cultural work upon yellow fever.

Source of Material—During the epidemic which prevailed for certain Gulf States in the summer and fall of 1922 we collected fresh material for study from selected cases at the fastigium of the disease. Blood carefully taken was immediately used. The reacting experimental animal supplied tissue as well as blood for cultivation tests.

[1] Ashburn, P. M., and Craig, C. F., *J. Infect. Dis.*, 1907, iv, 440.

[2] Duval, C. W., and Harris, W. H., *South. Med. J.*, 1924, xvii, 106.

[3] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.

Thus virus propagation was attempted directly with the blood of human cases as well as that of certain animals which had been inoculated with the human blood.

The materials used for cultivation were in most instances the filtered (Berkefeld) and unfiltered serum from blood that was allowed to coagulate, and the plasma obtained from citrated blood. Tissue cultures were prepared with the filtrate of macerated spleens from reacting animals. The quantity of inoculum employed for the individual culture tube was usually 1 mil.

Cultivation.—The special media used were the Smith-Noguchi (fragment of sterile rabbit kidney in ascitic fluid) and the Noguchi semisolid plasma mixture for leptospira cultivation. They were sterilly prepared and inoculated by means of a long sterile capillary pipette, one whose capillary portion reached readily to the bottom of the tube without admitting the bulb. The content of the inoculating pipette was introduced slowly into the nutrient from below upward by gradual pressure upon the rubber teat as the pipette was withdrawn. The medium was then capped with a layer of sterile paraffin oil in order to preserve partial anaerobiosis. Incubation was carried out at a temperature of 37°C. All tubes showing marked turbidity within 2 or 3 days were discarded as contaminated.

The non-contaminated inoculated media show no visible indication of growth under 3 weeks, particularly for the initial generation cultures. The first indication of growth in the ascitic fluid nutrient consists of a faint clouding or opalescence around the bit of kidney, which in several weeks after its appearance gradually extends upward to within 2 or 3 cm. of the capping layer of oil. In the upper strata of the medium the clouding is more significant of microorganismal growth than it is in the lower part about the piece of tissue, where macroscopic growth is often obscured or indistinguishable from the cloudiness produced by the disintegration of the contained piece of tissue. From the ocular view-point it is only the opalescence in the upper portion of the medium, which in the undisturbed tubes has gradually ascended from below, that we could regard as definite macroscopic evidence of growth. While at no time did we observe colony formations in the ascitic fluid, sedimentation and settling of the growth occurred after further multiplication had apparently

ceased, leaving now the previously clouded upper stratum as clear as that of the uninoculated medium.

On the other hand, the changes indicative of growth in the semisolid plasma medium are far more striking than those of the liquid ascitic nutrient. Here growth is first visible in the lower part of the tube, and appears as finely granular clouding which subsequently resolves into visible grains of pin-point size that are to be recognized as definite colonies. This character of growth gradually becomes pronounced in the medium until the upper limits of multiplication have been reached. The larger colonies are invariably found in the lower strata of the medium, becoming smaller and smaller until scarcely visible as the upper line of growth demarcation is attained.

When the number of colonies is large the individuals are so small as to give a diffuse cloudiness to the medium, and while separate colonies cannot be determined by the naked eye, the low power lens brings these distinctly to view. In cultures in which the colonies are fewer they are readily visible and may reach a diameter of 0.3 mm., appearing round and of a grayish color. Even under the most favorable growth condition distinct colonies are not visible in the semisolid medium before 3 weeks incubation for first generation cultures; however, they may appear earlier in cultures of subsequent transplants. The ocular demonstration of primary growth in the semisolid medium is exceedingly difficult and unconvincing when there is an absence of colonization.

Multiplication is extremely slow and takes place only under anaerobic conditions in media composed either of ascitic fluid or the plasma nutrient of Noguchi. No growth occurs in inoculated tubes of ordinary laboratory media under any conditions.

Berkefeld filtrates give rise to growth in the special media less constantly than the unfiltered material from which the filtered specimen was prepared. In the positive cultures obtained with filtrates the initial appearance of growth is much later, and when complete shows far fewer colonies; however, these attain a maximum size.

In a series of direct cultural experiments with the blood from twelve cases of dengue fever we have noted the identical growth in five instances. For another series of cultural tests with the blood from reacting guinea pigs that were previously inoculated with human

dengue blood, a similar growth was obtained. Likewise the blood from guinea pigs inoculated with recovered cultures yielded a growth of the same character. The cultures from the human and experimental animal behave alike with respect to growth features and seem in all particulars identical.

The earlier generation of cultures obtained directly from the human case induces in the guinea pig pyrexial and leucocytic response. Furthermore, the same reaction is occasioned in this animal with cultures recovered from the blood of the guinea pig.

Morphology.—The cultures from fluid and semisolid media observed with the dark field show innumerable dancing globular bodies which are single, in pairs, and in masses and not unlike, as far as arrangement and morphology, the "globoid bodies" described by Flexner and Noguchi[4] for poliomyelitis. They are devoid of independent motion and the individuals are not distinguishable from the colloid particles common in serum suspensions viewed with the dark-field microscope; a point of distinction, however, lies in the greater number of the minute organism and its arrangement in closely packed colony aggregations. While in a given microscopic field the majority of the globoid bodies occur singly, there are many diplococcoid forms. Short chains composed of four to six individuals are only occasionally encountered.

The microorganism is a minute coccoid body of quite regular form, measuring from 0.1 to 0.3 micron in diameter. It readily passes through the V and N Berkefeld filters. It cannot be stained by Gram's method, or by any of the simple dyes with the exception of carbolfuchsin. Even with this stain the organism is poorly colored. The various Romanowski methods of staining, particularly that of Giemsa, define more clearly the outline of the minute organism. Cultures from the human and guinea pig sources take the stains equally well. The difficulty with tinctorial methods employed is that the globoid bodies do not stain intensely and are not sharp in outline.

Considerable trouble may be experienced in recognizing the microorganism in stained preparations when these are prepared from cultures before visible colonization has occurred. This presumably is

[4] Flexner, S., and Noguchi, H., *J. Exp. Med.*, 1913, xviii, 461.

due to the paucity in numbers and the minute size of the organisms, making it hard to distinguish them from stained colloidal precipitates of the medium. Either nothing whatsoever can be recognized as microorganisms or merely globoid bodies the nature of which is indeterminate. It is in numbers one is convinced that he is dealing with a living structure. Before we obtained colonization in our cultures we did not suspect the nature of the often observed pairs, short chains, and masses of globoid bodies in the dark-field and stained preparations. The minute microorganism has cultural, morphological, and tinctorial features in common with the cultivated virus of poliomyelitis described by Flexner and Noguchi[4] as globoid bodies.

Animal Inoculation.—To determine whether the peculiar minute organism recovered in pure culture from human dengue blood and the blood of reacting animals possesses pathogenic properties, and is in this respect alike for the various cultures isolated from different sources, guinea pigs of approximately 250 gm. weight were used in the tests. The animals were injected intraperitoneally with 1 mil quantities of culture filtrates. In each instance the filtrate was tested for ordinary bacterial contaminators by inoculating portions of it into dextrose bouillon. The experiments were carried out for cultures of different sources as well as for different generations of the same strain. Only filtrates of cultures were employed for animal inoculation.

Experiment 1.—("Eber" strain. Recovered directly from human case (No. 11) in semisolid plasma medium. Culture of third generation.) Six guinea pigs, normal with respect to temperature and circulating leucocytes, each received intraperitoneally 2 mils of a filtrate from an 18 day old culture. There was macroscopic evidence of growth for the culture employed as indicated by a distinct cloudiness of the medium, most marked in the lower third of the tube. 4 to 6 days after the inoculation three of the pigs developed suddenly a temperature which ranged between 104°F. and 106°F., lasting from 3 to 5 days, when it subsided and remained normal. There occurred for each of the animals with pyrexia a concomitant drop in the circulating leucocytes which remained low for several weeks after the temperature became normal and the animal had apparently recovered. Aside from the pyrexia and leucopenia the animals showed no other signs of infection. Even during the height of the fever they did not seem ill to all outward appearances.

One of the pigs was sacrificed at the fastigium of the temperature and a portion of the filtered defibrinated blood was cultured to several tubes of special media and another portion injected into other animals including the monkey and rabbit. Globoid bodies identical to the microorganism used for inoculation were re-

covered in certain tubes of the special media which had been inoculated with blood of the reacting animal. Likewise some of the inoculated animals reacted in due course, excepting the monkeys and rabbits. In these species we at no time detected signs of infection.

Experiment 2.—("Eber" strain, from Case 11. Culture in sixth generation.) Four guinea pigs were injected intraperitoneally with 1 mil doses of the filtered culture. The inoculated dextrose broth controls for extraneous bacteria remained sterile. Two of the pigs developed temperature and leucopenia 4 to 5 days following the injection, and ran the usual course noted for the reacting animals. One of the pigs, however, developed a secondary rise in temperature which lasted for 3 days, after a 36 hour remission of the primary pyrexia ("saddleback" temperature curve). Both reacting pigs were bled from the heart during the height of the infection, the withdrawn blood defibrinated and the serum filtered and inoculated into other animals of the species. A portion of the filtrate was transferred to special media for *in vitro* cultivation. Of the inoculated culture tubes growth appeared in three out of eight after an incubation of 4 weeks at 37°C.

A remarkable feature for the growth in the semisolid plasma mixture was definite colonization, the colonies appearing as tiny grain-like particles in the lower two-thirds of the culture tubes. Microscopic examination of specially stained preparations showed these colony masses to be minute coccoid bodies identical to those in the culture used for the animal inoculation.

Experiment 3.—("Wetmore" strain. Recovered directly from the human case (No. 3).) Eight animals were inoculated intraperitoneally with 1 mil quantities of the filtered culture (first generation) of ascitic tissue medium which had incubated at 37°C. for 24 days. Two of the pigs reacted in a characteristic manner, the others failing to show any rise in temperature or fall in the circulating leucocytes during the period of observation. In one of the pigs the fever rose suddenly, 4 days after the inoculation, to 105°F., remaining around this level for 3 days, then remitting, to be followed by a rise of short duration. In this animal the leucocytes fell to 4,000 from an original count of 15,000 before inoculation. The pig was sacrificed on the 8th day of the infection. At autopsy the spleen was found greatly enlarged but otherwise nothing else noted that was remarkable. Blood was transferred to special culture media and also inoculated into other animals. No growth of coccoid bodies was obtained upon the special media because the blood contained a bacillus of the enteriditis group.

Experiment 4.—(Guinea pig strain. Recovered from animal reacting to injection of dengue blood from Case 11. Culture in sixth generation.) Four guinea pigs each received intraperitoneally 1 mil doses of the culture filtrate, which proved sterile in broth with respect to common bacteria. Two out of the series of animals injected developed pyrexial and leucocytic reactions 5 to 7 days following. In one pig the temperature reached 107°F. on the 3rd day of the reaction, and the circulating leucocytes fell to 3,000. Two of the animals, while failing to show temperature rises, developed well marked leucopenia. One of the pigs in the

course of the infection presented the so called "saddleback" temperature curve. At the height of pyrexia the animals were bled from the heart to obtain material for culturing and further animal transmission. Positive cultures were obtained from the blood filtrate upon plasma medium, and tinctorially and morphologically were similar to the culture used for injection and also the culture which had been isolated directly from the human case ("Eber" strain).

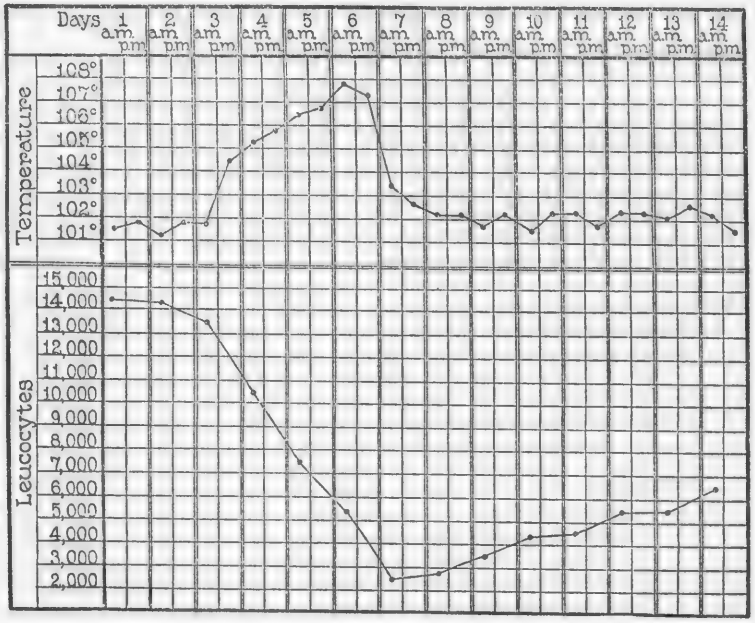


FIG. 1.

Text-fig. 1 is representative of the reaction in guinea pigs induced by the intraperitoneal inoculation of culture filtrates.

The experiments show that the inoculation of culture into the guinea pig is characterized by a primary pyrexia after a definite incubation period, with a secondary rise in temperature following a short period of remission and a concomitant fall in the circulating leucocytes. The reaction induced in the animal is alike for the culture filtrates of human and guinea pig sources respectively. Furthermore, the reac-

tion to culture inoculation is identical to that produced in the animal with fresh human dengue blood. Filtrates from cultures of the sixth subtransplant infect equally as well as those of earlier generations; however, for cultures further removed larger doses are necessary and fewer animals react. The monkeys and rabbits inoculated with cultures give rise to no pyrexia or fall in the circulating leucocytes. Negative results in these animals were also obtained with the blood from the human dengue patients.

DISCUSSION AND SUMMARY.

We have described the cultivation of a minute organism, upon the special media devised by Noguchi for growing spirochetal organisms, that gives rise to pyrexial and leucocytic reactions in the guinea pig. The reaction in the animal following the injection of culture filtrate is identical with that induced by the inoculation of human dengue blood.[5]

While it is possible, it is hardly probable that the infection of animals with cultures of this microorganism several generations removed from the original is due to a mechanically transferred virus with which the visible microorganism cultivated became accidentally associated. Furthermore, it is unlikely that such a virus would remain viable and be carried over to subcultures in sufficient numbers to infect the animal.

The microorganisms appear in culture as globoid bodies measuring from 0.1 to 0.3μ in diameter and are arranged singly, in pairs, and in short chains. They readily pass through the Berkefeld filter (N and V) and the filtrate gives rise to a characteristic reaction in the inoculated guinea pig. The filtrate yields in subplants the same globoid bodies of the original culture.

Initial cultures have been obtained directly from the blood of the human case as well as from the blood of guinea pigs reacting to the human material. However, only early generations retain the degree of virulence necessary to cause the experimental reaction, the culture of remote generation failing to infect.

As far as known this minute organism has characters in common with the globoid bodies of Flexner and Noguchi obtained from cases of

[5] Harris, W. H., and Duval, C. W., *J. Exp. Med.*, 1924, xl, 817.

poliomyelitis. In plasma semisolid medium the striking feature indicative of growth is colonization, which in itself serves to differentiate the opalescence of the medium occasioned by disintegration of the contained tissue.

As regards the presence of spirochetal organisms in the blood of dengue fever, we may state that examination was made of the material from human cases and from a large number of inoculated animals. Despite the most careful search with the dark-field microscope and repeated examinations of blood specimens stained by the best methods, we have been unable to find any demonstrable spiral organism in the blood of dengue patients or in the experimentally inoculated animal.

The minute organism herein described is frequently present in cultures of the blood of human dengue and of animals inoculated with dengue material. The inoculation of the culture into guinea pigs produces a response comparable to that occurring in the human case, and that induced by the injection of human dengue blood in guinea pigs. While we are of the opinion that the anaerobic globular bodies described may bear etiological relationship to dengue, we realize that further proof and confirmation of our work are required to establish the connection.

EXPLANATION OF PLATES.

PLATE 36.

FIGS. 1 to 5. Photomicrographs of globoid bodies in pure culture which were isolated directly from the blood of dengue patients upon semisolid plasma medium. Note the coccoid form of the microorganism and its arrangement singly, in pairs, and in occasional short chains. Magnification × 2,000. Zeiss obj. 2 mm.

FIG. 6. Two pairs of streptococci are shown for comparative size. Magnification × 1,000. Zeiss obj. 2 mm.

PLATE 37.

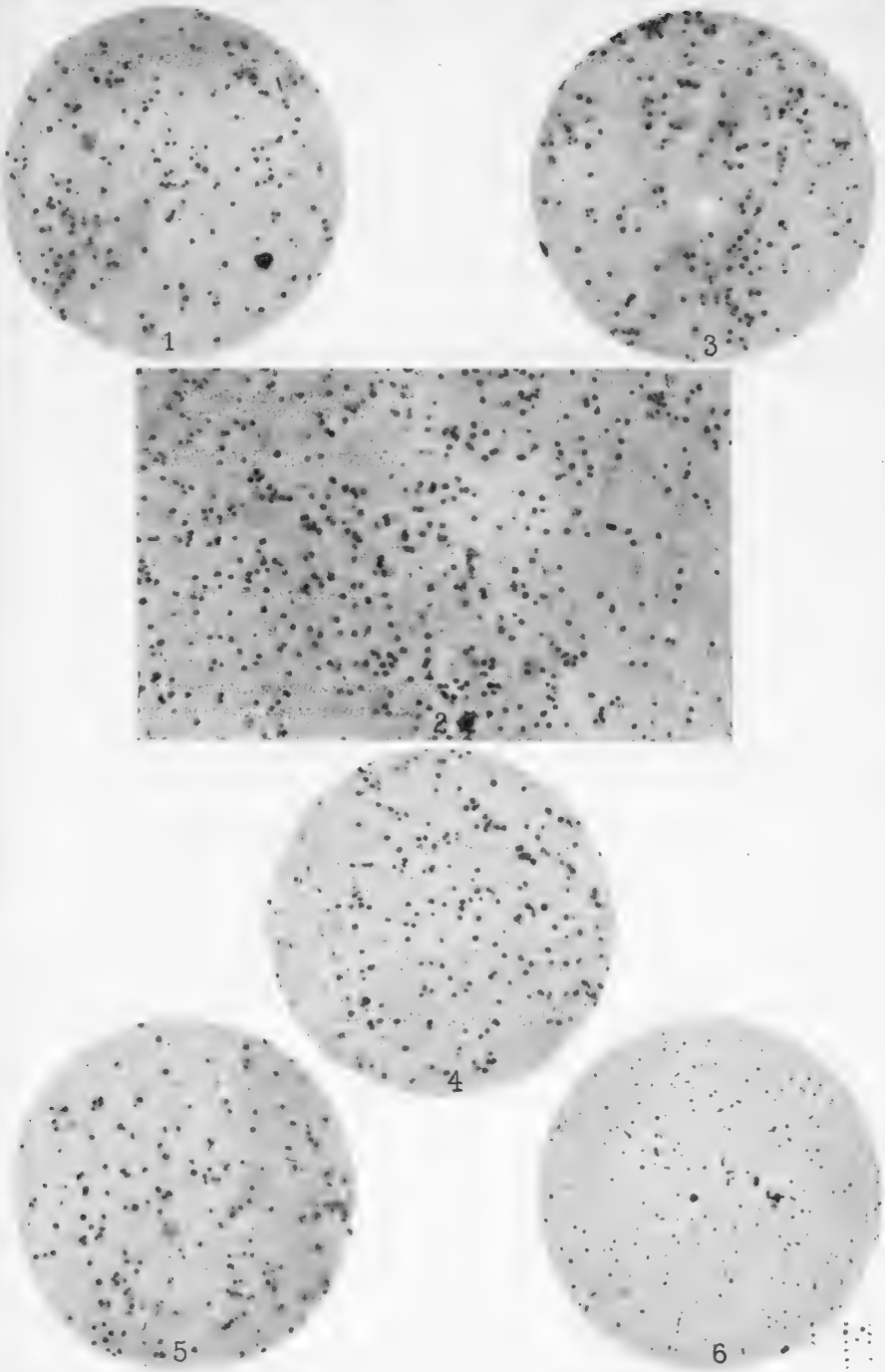
FIGS. 7 to 11. Photomicrographs of globoid bodies from pure culture with *Streptococcus viridans* to show comparative size and difference in intensity of staining. Fig. 7 shows one chain of seven and three pairs of streptococci; Fig. 8, one chain of four and two single streptococci. Figs. 9 and 10 show only globoid bodies; Fig. 11 contains one chain of four streptococci. Note that the globoid microorganism is much smaller and less intensely stained than the streptococcus. Magnification × 1,000. Zeiss obj. 2 mm.

FIGS. 12 and 14. Photographs of adrenal and spleen of guinea pig reacting to the virus of dengue fever.

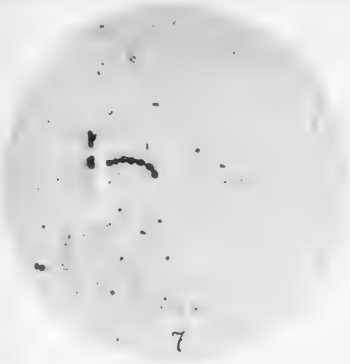
FIGS. 13 and 15. Normal adrenal and spleen of same weight animal for comparison. Note the great increase in size of the experimental dengue organs.

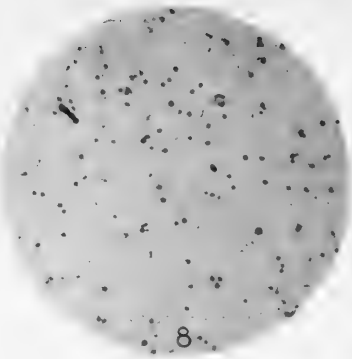
PLATE 38.

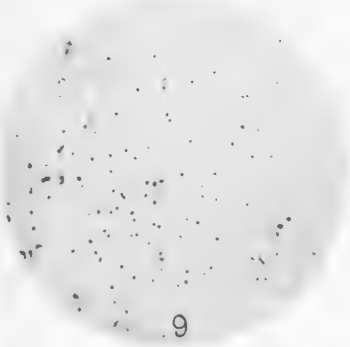
FIG. 16. Drawing of macroscopic growth of globoid bodies in anaerobic tubes of semisolid plasma media. Note *colonization* of culture in the form of minute grain-like particles more numerous and larger for lower stratum of medium, and ending rather abruptly 1 to 2 cm. below the covering cap of paraffin oil.

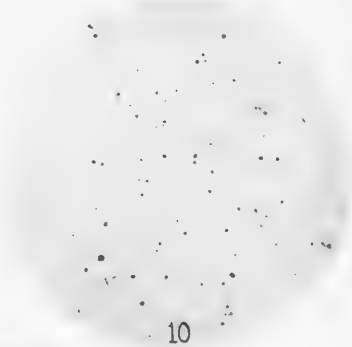


(Duval and Harris: Etiology of dengue fever. II.)









(Duval and Harris: Etiology of dengue fever. II.)

(Duval and Harris: Etiology of dengue fever. II.)

STUDIES ON THE MATURATION OF MYELOBLASTS INTO MYELOCYTES AND ON AMITOTIC CELL DIVISION IN THE PERIPHERAL BLOOD IN SUBACUTE MYELOBLASTIC LEUCEMIA.

By F. R. SABIN, M.D., C. R. AUSTRIAN, M.D., R. S. CUNNINGHAM, M.D., AND C. A. DOAN, M.D.

(*From the Department of Anatomy of the Johns Hopkins University, Baltimore.*)

PLATES 39 AND 40.

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INTRODUCTION.

Through the courtesy of the Medical Department of the Johns Hopkins Medical School and of the Hebrew Hospital and Asylum of Baltimore, we have had the privilege of studying the blood in several cases of leucemia during the past 2 years. The case which forms the basis of this report was from the Hebrew Hospital, and the clinical history has been contributed to this article by its medical director, Dr. Charles R. Austrian.

With the introduction of a new technique, by which differential studies of living blood cells can be made, it is of the utmost value to study the blood of leucemic patients from the standpoint of establishing the morphology of the blood-forming organs on such a basis as to open up an experimental attack on the mechanism, first, of the regulation, of the normal proportion of different types of cells in the circulating blood and, second, of the alteration in disease of the proportion and indeed of the types of cells appearing in the blood.

In the case reported here, we think that we were able to identify myeloblasts with the supravital technique, to demonstrate that the maturation of myeloblasts into myelocytes followed the transfusion of whole blood, and to identify the stages through which myeloblasts develop into myelocytes and finally into leucocytes. Also we saw in these living preparations some remarkable types of nuclear and cellular division, which must ultimately be taken into consideration in the analysis of the cause of leucemia.

Technique.

For the discrimination of myeloblasts in the supravital technique we used neutral red and Janus green.[1] The method already described by Sabin (5) was followed, but in general for the blood of leucemic patients or for smears from bone marrow, in which there were many more white cells involved, it was found that the strength of both dyes should be about twice the amount for normal blood. In regard to the use of Janus green three different reactions have been observed. When the dye was in the correct proportion for staining the cells without apparent injury the mitochondria were found colored a brilliant blue and certain substances in the platelets stained a dull green; but when the concentration of the dye was too great the cytoplasm of the young white blood cell, which is known to be strongly basophilic, showed a diffuse grey tone.

We are indebted to Mr. James F. Didusch, artist of the Department of embryology of the Carnegie Institution of Washington, for the drawings of the living cells.

For the case which is the basis of this report we have the following clinical history and pathological report.

Clinical Report.

C. M., a white youth aged 15 years, was admitted to the Hebrew Hospital Nov. 23, 1923 (Med. No. 2217), complaining of "weakness and constant vomiting." His family and past histories were negative. In June, 1923, 6 months before he came under observation, he noted a sense of weakness, and was frequently nauseated and vomited. In Sept., 1923, he had dysphagia and an exudative inflammation of the tonsils without fever. 3 weeks prior to admission to the hospital, "sore throat" recurred, and a week before symptoms of pharyngeal inflammation recurred but no epistaxis or stomatitis. Asthenia, vomiting without hematemesis or melena, dyspnea, and acroparesthesia developed during the past week and became progressively more distressing. The body weight decreased from 120 to 94 pounds in the 6 months before admission.

Physical Examination.—The patient is emaciated, obviously weak, and with striking pallor without icteric tint. No hemorrhagic spots and no areas of infiltration are present in the skin or mucous membranes. There is no exophthalmos and though there is anisocoria the pupils are regular and react normally. The ocular fundi show abnormal fullness of the retinal veins, slight edema of the nerve heads, and on the right side a small diamond-shaped hemorrhage. The tongue is heavily furred. There is slight gingivitis; the tonsils are enormous.

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[1] The preparation of Janus green put up by the National Aniline and Chemical Company gives especially brilliant results. We use the Grübler vital neutral red (*n.* Ehrlich).

No pharyngeal or tonsillar ulceration or exudation is present. There is a general enlargement of the lymph nodes. The lungs are clear and there is no evidence of a mediastinal mass. The heart is not enlarged. There is slight tachycardia; no arrhythmia. The first sound at the apex is not abrupt and the short systolic murmur accompanying it, though audible at the apex and transmitted into the axilla, is of maximum intensity in the second left interspace and the second pulmonic sound is not accentuated. The pulse is 108 per minute, regular in force and rhythm, of fair volume and low tension. There is no sclerosis of the peripheral arteries. The abdomen shows nothing abnormal except that the edge of the spleen is just palpable. There is no edema. Neurological examination is negative.

Laboratory Examinations.—Blood examinations are shown in Table I.

Fragility test: Hemolysis begins in hypotonic solution 0.42 per cent—complete in solution 0.26 per cent.

(Control): Hemolysis begins in hypotonic solution 0.42 per cent—complete in solution 0.28 per cent.

Urinalysis.—Clear amber, acid, albumin 1 plus, no sugar, acetone, diacetic acid, or urobilin. Urobilinogen trace. Microscopic examination: numerous granular casts; red blood cells and epithelial cells, few white blood cells.

Chemical Examination of Blood.—Urea nitrogen, 15.12 mg. per 100 cc. Urea, 32.3 mg. Uric acid, 3.2 mg. Creatinine, 1.5 mg. Carbon dioxide, 28 volumes per cent. Wassermann reaction—negative. Blood grouping—Group IV.

Spinal Fluid.—Clear, colorless, under increased pressure. Cells, 5 per c.mm. Wassermann reaction—negative (0.2 and 1 cc.). Globulin—not increased. Colloidal gold and gum mastic curves—negative.

Gastric Analysis.—Ewald test meal removed after 30 minutes. Small amounts of fluid material with well digested particles recovered. No gross blood, mucus, or pus. Free hydrochloric acid, 10 acidity per cent. Total acidity, 18 acidity per cent. No lactic acid. Microscopic examination negative. Stool examination negative. No ova, parasites, pus, or blood.

The history of the patient, the clinical manifestations, and the increase in the number of white corpuscles with the large number of immature cells established the diagnosis of acute leucemia. The extent of the adenopathy and the predominance of cells, apparently of the lymphocytic series in the blood first examined, indicated an acute lymphatic leucemia but this view was modified by all subsequent studies of the blood and a diagnosis of subacute myelogenous leucemia was the final one.

Clinical Course.—During the next 6 weeks in spite of intensive treatment with neoarsphenamine, sodium cacodylate, Blaud's mass, and two transfusions of blood, the progress of the disease was unarrested. The superficial lymph nodes and spleen enlarged. Epistaxis occurred on Dec. 1, hemorrhagic spots appeared in the mucous membrane of the lips, and retinitis became more extensive, hemorrhages large and small appearing in the retina of both eyes. These

TABLE I.

Differential Counts, from Fixed Smears.

Date.	Red blood corpuscles.	Hemoglobin.	Color index.	White blood corpuscles.	Differential count—200 cells.						Oxidase.	Remarks.
					Polymorphonuclear neutrophils.	Polymorphonuclear eosinophils.	Polymorphonuclear basophils.	Lymphocytes.	Monocytes.	Path cel's.		
		per cent			*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
Nov. 23	1,416,000	25	0.85	81,550	3.5	0	0	0	0	95.5		Bleeding time 3 min. Coagulation time 3.5 min. Anisocytosis—poikilocytosis.
" 26	1,368,000	25	0.9	111,300	1.5	0	0	15.0	0	83.5		
" 28												Blood CO_2 40 volumes per cent.
" 29	1,304,000	25	0.9	152,450	1.0	0.5	0	9.0	7.5	82.0		Myeloblasts and normoblasts.
" 30											6.0	Many myeloblasts. Some with dividing nuclei.
Dec. 1	1,216,000	23	0.9	160,000	0.5	0	0	3.0	0	96.5		
" 2											8.0	
" 3	1,048,000	20	0.95	108,250	6.0	0	0	18.0	0	76.0	9.25	Red cells show polychromatophilia and anisocytosis.
" 5	764,000	17	1.3	195,000							12.0*	Intravenous injection of 400 cc. of citrated blood from his mother.
											12.0†	
" 6	1,512,000	26	0.88	110,700	4.0	0	0	23.0	0	72.0		Red blood cells less pale. Myeloblasts 15, normoblasts 13.
" 7											55.5	
" 8	1,192,000	20	0.9	109,450							46.0	

Dec. 10											46.25	
" 11	1,440,000	20	0.8	134,550							42.5	
" 12											46.5	
" 13	1,506,000	19	0.6	162,200							52.6	
" 14											52.5	A second transfusion of 500 cc. citrated blood.
" 15	1,928,000	23	0.6	177,400							58.5	
" 17	1,600,000	25	0.8	230,400							58.6	
" 18											68.0	
" 19	1,680,000	20	0.65	308,000							60.0	

* Count made before transfusion.

† Count made after transfusion.

were the only evidence of a hemorrhagic tendency. Puffiness of the eyelids and edema of the dependent parts were noted on Dec. 5. There was never tenderness of the bones. Occasional pain in the abdomen, nausea, and vomiting increased the discomfort of the patient, but there were no intestinal disturbances. On Dec. 5, small areas of ulceration developed on the gums. Superficial, white, circumscribed, and only slightly infiltrated at first, these areas slowly increased in size, were covered by a yellowish exudate, caused much pain and marked fetor oris. By Dec. 15, the mucous membrane of the entire roof of the mouth was necrotic. Bacteriological studies of the buccal lesions showed no fusiform bacilli but streptococci and staphylococci. Concomitantly with the extension of the buccal lesions, the submaxillary and submental lymph nodes increased in size.

The circulatory disturbances increased during the 2nd week of observation; he had increasing dyspnea, a sense of substernal tension, edema, throbbing of the peripheral arteries, and capillary pulsation, a to-and-fro hum over the cervical veins, and systolic and diastolic murmurs over the precordium were heard by Dec. 14. There were no signs of increasing cardiac dilation. The systolic blood pressure varied between 125 and 105 mm. Hg and the diastolic pressure was 50 mm. or so low it could not be determined. The output of urine varied between 450 and 2,300 cc. and, after the first examination, urinalysis showed no abnormalities except albuminuria. The entire course in the hospital was febrile. The fever was remittent between 99.6° and 101.6°F. to Dec. 1, after which the general level was higher, the maxima varying between 102.2° and 105°F. with daily remissions of from 0.5–4°F. The more marked pyrexia accompanied the spread of the necrosis in the mouth. There were no chills but sweating was profuse. The pulse rate varied with the variations of temperature but the rate was never slower than 100 beats per minute. Except for dyspnea of short duration on Dec. 5, and polypnea during the last 10 days of the illness, there were no respiratory disturbances. After Nov. 23, the patient was dull mentally and apathy gradually increased. Though there were short intervals after the transfusions when the patient seemed more alert, lassitude was replaced by somnolence during the week of Dec. 10, but the patient was never comatose. Several blood cultures were sterile. X-ray examination of the chest showed enlargement of the mediastinal shadow. The patient died on Dec. 20; he had increasing dyspnea but was conscious until the end. The autopsy was performed by Dr. Arnold Rich of the Department of Pathology of the Johns Hopkins Medical School, who gave the following report.

Pathological Diagnosis.—Myeloblastic leucemia with green chloromatous coloration of all lymph glands and of all macroscopic foci of myeloblastic infiltration but without any isolated chloromatous tumor. Myeloid hyperplasia of bone marrow. Splenomegaly. Generalized enlargement of lymph glands and solitary lymph follicles of digestive tract. Extensive myeloid infiltration of all lymphoid tissues, spleen, liver, kidneys, adrenals, epicardium, lungs, retroperitoneal tissues, dura mater, testis, and choroid coats of the eyes. Infiltration of intestinal mucosa with ulceration. Slight bilateral hydroneph-

rosis from pressure of enlarged lymph glands upon ureters. Petechial hemorrhages in skin and retina. Terminal aspiration of stomach contents. Pulmonary edema. Ulceration of gingiva.

Report of the Vital Studies on Blood Cells.

Myeloblasts.—The first supravital differential count of the blood of this case is given in Table II.

The predominating cell we diagnosed a myeloblast. Our criteria for the discrimination of this cell were largely obtained from the study of a fatal case of myeloblastic leucemia from Dr. Longcope's clinic in the Johns Hopkins Hospital (B. R., Med. No. 50043, Path. No. 7716). From this case the cell of Fig. 1 was drawn. This was a cell of intermediate size whose cytoplasm contained great numbers of tiny mitochondria; nothing in the cytoplasm reacted to neutral red. The nucleus was large in proportion to the cytoplasm. The cell of Fig. 2 was from another case of myelogenous leucemia (J. F. F., Med. No. 50256), Johns

TABLE II.

Vital Differential Count of 300 Cells, November 27.

	per cent		*per cent*
Myeloblasts	92.33	Lymphocytes, small	0.66
Myelocytes	2.33	" intermediate	0.33
Polymorphonuclear neutrophils, very sluggishly motile or with some movement of granules and no locomotion	2.66	Giant cells	1.33

Hopkins Hospital. In this case, the abnormal cells had many mitochondria but in addition every cell had a clump of particles that stained in neutral red as do the neutrophilic granules. This type of cell we have identified as the earliest form of the myelocyte and have designated it as *myelocyte, Type A.* In some experimental work on the bone marrow of pigeons and rabbits, we (1) have identified similar cells as myeloblasts and early myelocytes.

In the study of the case of C. M. from the Hebrew Hospital the cell shown in Fig. 3 was the type we have identified as the myeloblast. In regard to the rest of the cells in the differential count, the small lymphocytes were wholly typical; and the one intermediate lymphocyte showed the characteristic type of motility (5). Throughout the course of the case there were about 2 per cent of small lymphocytes and at autopsy the lymph glands, although almost wholly transformed into myeloid tissue, nevertheless showed a few typical lymphoid

follicles. The myelocytes in this count were of the type shown in Fig. 8, which is the form that precedes the stage of the leucocyte. We made the diagnosis of myeloblastic leucemia on the presence of 92.33 per cent of cells which we identified as myeloblasts and on the myelocytes. The giant cells which were occasionally present were probably clasmatocytes.

The majority of the myeloblasts were like the one shown in Fig. 3. This cell had a diameter about twice that of a red blood cell. In general the myeloblasts throughout the course of the disease could be divided into three groups; a very large cell, not shown on our plates, whose diameter was two and a quarter times the diameter of the red cell, intermediate types with a diameter nearly twice that of a red cell, and small cells like that of Fig. 11, with a diameter about the same or less than that of a red cell. In proportion there were always fewest of the small types; for example, on December 5, in a count of 100 cells taken 2 hours after the transfusion there were 79 myeloblasts, of which 2 were small, 50 intermediate, and 27 large; while on the next day taking myeloblasts and myelocytes together there were 8 small, 37 intermediate, and 48 large types in 100 cells.

It seems to us, therefore, that the myeloblast is a cell that has a cytoplasm containing nothing that reacts to vital dyes except many small mitochondria. The nucleus is large, contains a coarse chromatin network and one or more definite nucleoli. It is usually placed centrally but may become as eccentric as the nucleus of the very early myelocyte of Fig. 5. Such myeloblasts are illustrated in Figs. 1, 3, 9, *a*, 9, *b*, 10, *a*, 10, *b*, and 11.

In some experimental work on the bone marrow of birds and rabbits, we (1) have found a cell which we consider to be the progenitor of all the white blood cells. It was impossible to identify this cell in normal bone marrow but in bone marrow which had been so simplified by experimental procedures that no cells remained between the vessels except the fat cells and the undifferentiated mesenchyme or reticular cell, it has been possible to follow the differentiation of such a *primitive white cell.* The reticular cell contained nothing in its cytoplasm that reacted to vital dyes, while the primitive white cell had a more basophilic cytoplasm which, when vitally stained, showed a few large, scattered mitochondria. The myeloblast developed from this cell by an increase in the number and a decrease in the size of the mitochondria. These cells are illustrated in the article quoted above (1).

When myeloblasts are stained with Wright's or Wilson's stain, in the usual concentration, the cytoplasm proves to be uniformly basophilic. For the study of myeloblasts in smears of leucemic blood, or for the study of the cells in bone marrow, we have found it best to dilute the original stain with equal parts of methyl alcohol.[2] In the differential counts made with fixed smears in this case there was difficulty in discriminating small myeloblasts from small lymphocytes and reference to the counts in the clinical history will show that they could not be discriminated with the use of concentrated stain. The fact of the amitotic division in a small cell such as the one shown in Fig. 11, the small myeloblast, indicates that the supravital technique had an advantage. With dilute Wright's stain the myeloblasts had a basophilic cytoplasm and many of them showed a small acidophilic spot beside the nucleus, a possible acidophilic centrosphere. This spot did not show at all in the living cells. It gives an additional point in the discrimination of myeloblasts from lymphocytes. Cells with such a pale spot in the cytoplasm are shown in Plates xiiia and xiv of the Atlas of Pappenheim and are identified by him as *Lymphoidocyten*. On his Plate xiiia, the entire range of cells to be considered as myeloblasts is shown, those in the fourth and fifth rows all having centrospheres, while Cells 1, 3, and 17 in Plate xiv are also typical myeloblasts. Thus one of the types of cells called by Pappenheim the *Lymphoidocyte* we identify as the myeloblast.

Myelocytes.—In following the case of C. M. we were able to trace the development of myelocytes from myeloblasts. The first step in this transformation was the development in the cytoplasm of a small clump of neutrophilic granules or their precursors, Fig. 4. These granules stained more intensely in the neutral red than the neutrophilic granules of the adult leucocytes and it is well known that in smears of blood in Wright's stain the neutrophilic granules of the early forms have a bluish tint in contrast to the pink shade of neutrophilic granules in leucocytes. This difference is well shown by Pappenheim and it is on this evidence that we refer to the first granules that stain with neutral red as the precursors of the neutrophilic granules. Pappenheim called these cells *Lymphoidocyten mit myeloischer Azurkörnung*. Since we could not see the centrosphere in the living cell we could not decide definitely that the neutrophilic granules had no relation to it but it was clear that the original clump of granules did not make a rosette around it. This development in the cytoplasm of granules that stained with neutral red was the first evidence of neutrophilic

[2] We use the methyl alcohol put up especially for blood stains by the National Aniline and Chemical Company.

granulation and we have designated cells having a small clump of not more than ten to fifteen such granules, as *myelocytes, Type A*. We are showing three cells of this type in Figs. 2, 4, and 5. They correspond to Cell 4 on Plate xiv of Pappenheim's Atlas. In his figure it can be seen that the entire clump of granules is practically identical with that of those staining in neutral red as shown in our Figs. 2 and 4. In the cell of Fig. 5, it will be seen that there was a group of four granules, which stained with neutral red, in a large mass of mitochondria on one side of the nucleus and three neutral red granules on the other side. We have classified this cell with the myelocytes, Type A, on account of the small number of neutrophilic granules, and because the arrangement of the mitochondria is that of the myeloblast. It might be taken as a transition toward, or as the earliest phase of, the next stage in which the neutrophilic granules become dispersed throughout the cytoplasm. Such a cell brings up the question as to how constant an original clump of the neutrophilic granules is, and whether it is the rule rather than the condition in which the granules are diffusely scattered from the start. We consider that the clump of granules represents the more typical early form, first on account of the greater number of types like that of Fig. 4, and second on account of certain observations given under the subject of the oxidase reaction.

The second stage in the development of the myelocyte is shown in Figs. 6 and 7, which we call *myelocyte, Type B*, and which consists in the increase of neutrophilic granules, with a decrease of mitochondria. This decrease in mitochondria may not be in inverse proportion to the increase in neutrophilic granules, but it is evident that when the granules that react to neutral red become numerous the mitochondria are markedly reduced. In the living cell it was possible to see how the cells of Type A became Type B because there was a slow movement of the individual neutrophilic granules in the cytoplasm so that the original clump of granules became dispersed and intermingled with the mitochondria. Throughout the second stage no pattern whatever could be made out either of the mitochondria or of the neutrophilic granules.

In the cells of Figs. 6 and 7, neutrophilic granules and mitochondria were in about equal proportions. The phase which we have called

Type B must be considered as including every possible variation in the proportion of neutrophilic granules to mitochondria, between the two extremes which we have called Type A, with the first small clump of neutrophilic granules and masses of mitochondria, and the cells of Type C, with a maximum of neutrophilic granules and a minimum of mitochondria. A group of cells which we should term myelocytes, Type B, is shown in the first two rows of Pappenheim's Plate xiiib, and on Plates xiiic, and xiv. By comparison of these three plates of Pappenheim it will be seen that the cytoplasm of the cells in this phase may be more or less basophilic. In the cases that we have studied, the cytoplasm of the cells in this phase was faintly basophilic.

The third stage of the myelocyte, or Type C, as we have termed it, is shown in Fig. 8; in this stage neutrophilic granules predominate and the mitochondria have been reduced to a narrow rim in the periphery in the cell. This is the type of myelocyte that predominates in normal bone marrow. The earlier leucocytes have a few mitochondria which are intermingled with the neutrophilic granules and move in the cytoplasm with them; such young leucocytes are occasionally found in the circulating blood but most of the leucocytes of the blood are without mitochondria.

In the transformation of myelocytes into leucocytes, we think that the well known changes in the nucleus preclude any further initiation of cell division; that there is usually an entire loss of the mitochondria from the cytoplasm; and that there is a decided change in the cytoplasm so that the cell becomes capable of ameboid movement. This last change involves a streaming of the cytoplasm correlated with movement of the cell. Indeed in the vital technique it becomes clear that both myeloblasts and myelocytes are cells without ameboid activity. In the myelocyte there may be no movement or a slight shifting of granules, but any flowing or streaming of the cytoplasm, carrying the granules along in the current, is to be seen only when the myelocyte is becoming a leucocyte. In the study of films of blood by the vital technique this lack of ameboid activity of the myelocyte is an important point in discriminating it from the leucocyte. Thus we have followed the changes which represent the maturation of myeloblasts through the stages of the myelocytes into leucocytes by the vital technique.

Maturation of Myelocytes.—In following this case we have given our results in two charts.

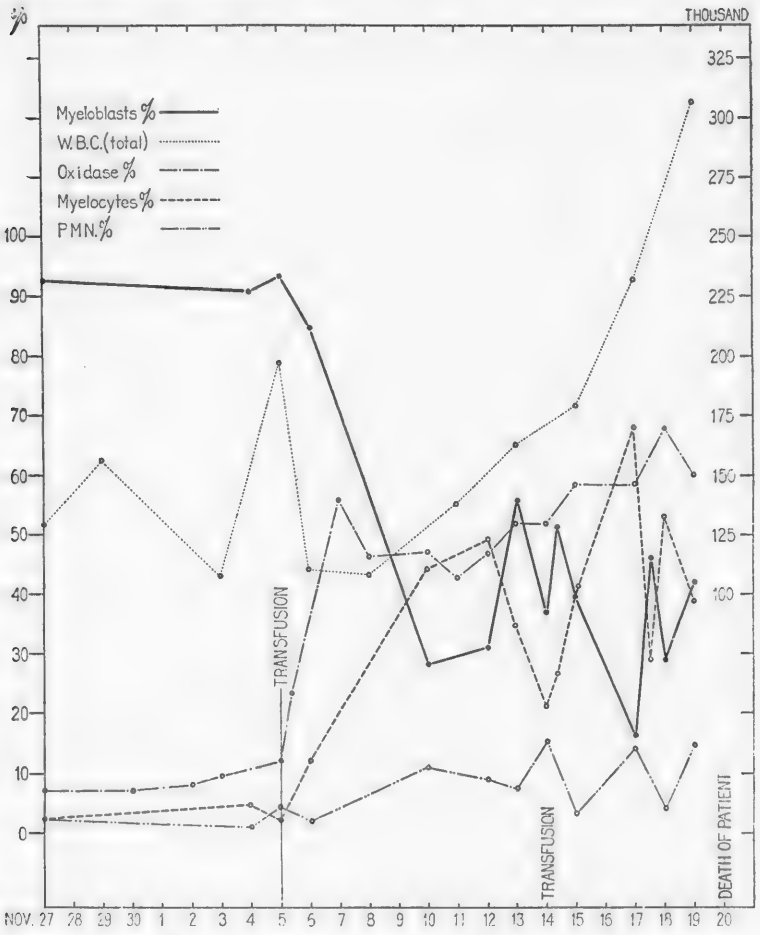

CHART 1. Graph of studies on the blood of C. M., Hebrew Hospital, Baltimore. The line of the total counts of the white blood cells is to be referred to the column of thousands along the right hand margin. The other data are given in percentage and represent myeloblasts, Types A, B, and C taken together, polymorphonuclear neutrophilic leucocytes, and the percentage of cells showing the oxidase reaction.

The first graph shows the total white count, the myeloblasts, the total myelocytes, the polymorphonuclear leucocytes, and the oxidase reaction. For the total white counts and the oxidase counts we are indebted to Dr. Ann Purdy, in charge of the clinical laboratory of the Hebrew Hospital; the rest of the data on the charts were taken from our vital counts. All of the data plotted on the charts are in terms of percentage except the one line of the total count of the white cells. This we found more feasible because our counts did not always coincide with the hours on which the total counts were made. The number of white cells was 81,550 on admission Nov. 23; our chart begins on the 27th, but the first few days are representative of conditions between Nov. 23 and Dec. 4. The total white count varied during the first few days shown on the chart, with a marked rise in the count on the day of the first transfusion, Dec. 5, reaching 195,000. The next day the number of cells returned to about the previous level, but from then there was a steady increase to a maximum of 308,000 on Dec. 19,

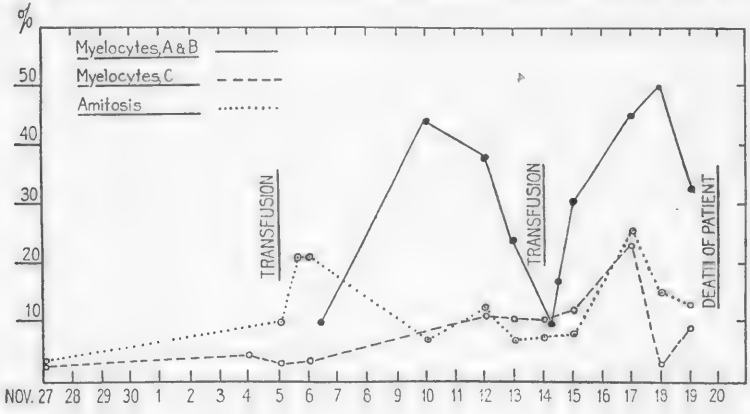

CHART 2. Graph of studies on the blood of the same case as Chart 1. The data represent the percentage of myelocytes, Types A and B together, in contrast to the myelocytes, Type C; and the percentage of cells showing amitosis.

the day before death. During the early days the myeloblast was the predominating cell with few leucocytes and few myelocytes. Throughout the course of the disease the leucocytes were mostly of the neutrophilic variety, but occasionally basophilic and eosinophilic leucocytes were seen, and one basophilic myelocyte, but these forms were too few to be considered in the chart. The curve of the myelocytes on this chart represents the combined number of all three types together.

Chart 1 shows that following the first transfusion there was a drop in the proportion of the myeloblasts accompanied by a slight increase in leucocytes and a marked increase in myelocytes. In other words,

there was a shifting of the cells toward more mature types. The effect was more marked after the second transfusion when the curve of the myelocytes went considerably above that of the myeloblasts, but the course of the chart indicates that this shifting of the types of cells reflected no actual gain in the nature of a clinical remission.

On the second chart we have presented three compilations, also given in percentages, the myelocytes, Type C, as compared with the more primitive forms, the myelocytes of Types A and B combined, and the cells showing amitosis. It will be recalled that on Nov. 27, the day of the first vital count, there were 2.33 per cent of myelocytes, Type C. We saw none of the myelocytes of Types A and B in the vital preparations until Dec. 5, the day after the first transfusion

TABLE III.

Vital Differential Count, 300 Cells, December 13.

	per cent		*per cent*
Polymorphonuclear neutrophils	7.3	Myelocytes, Type C	10.6
Myeloblasts	55.0	Lymphocytes	3.0
Myelocytes, Types A and B	24.0		

though there were a few cells of Type B identified in fixed smears. On the day of the first transfusion, Dec. 5, we made three vital counts, one before the transfusion, the others an hour, and 2 hours after. In the first count there were 93 per cent myeloblasts as shown on Chart 1, in the other two counts 77 and 79 per cent respectively. This difference was made up in an increase in leucocytes, "non-motile" cells, and damaged cells.[3] This immediate result we thought without special significance since it included the donor's blood and it has therefore not been shown on the chart; but on Dec. 6, we found three marked changes. First, the most significant change was that 10 per cent of the total white cells were myelocytes of Type A, which had appeared at the expense of the myeloblasts. This indicated that the myeloblasts had been stimulated to maturate into myelocytes. Second, though the percentage of the leucocytes did not increase markedly, being 2, 6, and 10 per cent respectively, in three counts, there was a decided increase in their motility. Third, there was an increase in the percentage of cells showing amitosis, Chart 2.

By the time of the second transfusion there had been another shift in the proportions of the myeloblasts and myelocytes, as will be seen in Table III.

[3] We now regard the name "non-motile cell" (5) as wholly inadequate, because the essential change is not from motility to non-motility, but in the appearance of the neutrophilic granules. They become highly refractive, swollen, and wholly resistant to vital dyes. In leucocytes, it is true, the motility ceases entirely, and both cell and nucleus become edematous; but in this study we have seen myelocytes, which of course have never developed the type of rapid ameboid activity characteristic of leucocytes, showing this same change in the nature of the neutrophilic granules.

On Dec. 14, counts were made a half hour and an hour and a half after the transfusion, as shown in Table IV. ½ hour after, there were 23 per cent of cells that we interpreted as old leucocytes, and thought that they had probably come from the donor's blood. They had a vacuolated cytoplasm and few neutrophilic granules and their nuclei did not look like the nuclei of the myeloblasts. In Table IV it will be seen that the blood taken 30 minutes after the transfusion

TABLE IV.

Vital Differential Counts, after the Second Transfusion, December 14.

	3.55 p.m.	4.45 p.m.
	per cent	*per cent*
Polymorphonuclear neutrophils	15	10
" " old, possibly from donor's blood	23	6
Myeloblasts	37	51
Myelocytes, Types A and B	10	17
" Type C	11	10
Lymphocytes	3	3
Giant cells	0	3

TABLE V.

Vital Differential Counts, December 15.

	11 a.m.	11.30 a.m.
	per cent	*per cent*
Polymorphonuclear neutrophils	5	3
" basophils	0	1
" eosinophils	1	0
" neutrophils, damaged	0	1
Myeloblasts	25	53
Myelocytes, Type A	5	13
" " B	29	14
" " C	11	14
Special phagocytic cells	21	0
Non-motile cells, leucocytes and myelocytes	3	0

was quite out of alignment with our other counts. We interpreted this as an irregular mixing of the blood of the donor and recipient and that the leucocytes marked old were those of the donor. It was quite striking that in an hour and a half after the transfusion (4.45 p.m.) the differential count was quite like those which had preceded. Again on the next morning there was one very irregular differential count, and two records taken a half hour apart are shown in Table V. On the first count of Dec. 15, the cells were markedly clumped and the group called "special phagocytic cells" was different from any others that were seen throughout the case. They had indented nuclei, their cytoplasm had fine gran-

ules equally distributed between mitochondria and neutral red bodies, but they showed marked signs of phagocytosis. Some had taken up red blood cells and others masses of debris. We saw these cells but once and were not sure of their identity; they were more like monocytes than any other type.

In general it will be noted on Chart 2 that, following each transfusion, there was a marked rise in the number of myelocytes of Types A and B. After the first transfusion the peak of these myelocytes was reached on the 5th day with a return on the 9th to almost the previous level. A second even higher peak was reached on the 4th day after the second transfusion, followed by a sudden drop on the following day, which was the day before the patient died. The greatest number of myelocytes of Type C occurred on the 3rd day after the second transfusion.

Oxidase Reaction.—The study of the oxidase reaction has proved interesting. We have used the Goodpasture (3) technique, which demonstrates the "peroxidase ferment."

From the first chart it is obvious that as the number of myelocytes increased at the expense of the myeloblasts, the proportion of cells positive to the oxidase reaction increased. In general, it will be noted that the entire oxidase curve runs a little low when correlated with the total myelocytes and leucocytes in vital counts. This we interpret as due to the loss of fragile cells in making smears We found in making the correlation between differentials on fixed smears and vital counts that all injured cells in fixed preparations must be included. On the other hand, it was well nigh impossible to make mathematically correct, differential counts of any of these preparations, especially during the latter part of our studies, because the number of white cells involved was so large.

In studying the slides stained by the oxidase method the cells showing the positive reaction could be divided into three groups; first, with one large blue granule in the cytoplasm; second, with fine scattered granules; and third, with massive granulations. The three types of cells classified by the oxidase granules corresponded so well with the three types of myelocytes outlined above, that it seemed feasible to consider cells having one large oxidase granule as myelocytes of Type A, those with scattered, fine, blue granules as myelocytes of Type B, leaving the cells with the massive oxidase reaction to correspond with the myelocytes, Type C, plus adult leucocytes.

In Table VI we have given the correlation between the vital counts and the oxidase counts on 4 different days. Note that the total vital count of all the myelocytes and polymorphonuclear leucocytes represents an estimate of what the oxidase reaction ought to be.

Each column represents 100 cells and it is clear that there is considerable variation in the counts of blood taken at the same time. The most interesting variation is in the vital counts of Dec. 18, in which it will be seen that the myelocytes, Type A, are far in excess of the corresponding cells with one oxidase granule

in the fixed smears; but the sum of the myelocytes of Types A and B agrees closely with the sum of the first two numbers in the oxidase count. We think that such a cell as that shown in Fig. 5 is an adequate explanation of such a discrepancy, for this cell would appear as a cell with the scattered granules in the oxidase reaction but as a myelocyte, Type A, in the vital count. It will be seen in Chart 1 that the correlation between the vital counts and the counts of the specimens stained to show the oxidase reaction is not exact, but, in view of the difficulties in making the counts, is suggestive enough to be regarded as presumptive evidence that the myeloblasts give a negative reaction to the oxidase stain; that cells with a small clump of neutrophilic granules or their precursors give one relatively large granule; that myelocytes of the intermediate phase of mingled neutrophilic granules and mitochondria give a positive oxidase reaction of fine, blue granules, while the myelocytes, Type C, give the same type of massive reaction as do the leucocytes.[4] It is entirely demonstrated that the maturation of myeloblasts into myelocytes was accompanied by an increase in the oxidase reaction. In following this case we had practically no monocytes to complicate the picture unless there were a few after the second transfusion. Thus from the sum of the myelocytes and leucocytes of the vital counts in this case we could give a fair estimate of the percentage of cells showing a positive oxidase reaction.

Amitosis.—One of the most striking features of the case was the amount of amitosis in the blood stream. There were also cells undergoing mitosis in the blood stream, since we found them in fixed smears, but we did not see any spindles in the living preparations.

We are showing in our figures five examples of amitosis drawn from the living preparations. The drawings shown in Figs. 9, *a* and 9, *b* were made on Dec. 4; when the cell, which was a large myeloblast, was first seen there were one large nucleus in the upper part of the cell and two small ones, widely separated, in the lower part. There was no question but that the three nuclei were distinct; one large and two small, unequal ones. By the time the drawing was started, 15 minutes later, the two small nuclei had approached each other and there was a slight constriction of the cytoplasm as shown in Fig. 9, *a*. From this time on, the entire process took 25 minutes. As shown in the drawings, the two small nuclei approached, fused, and then the cell divided, giving two cells of approximately equal size but with unequal nuclei.

The process by which nuclei fused in the cells of this case is shown better in Figs. 10, *a* and 10, *b*. When this cell was first seen it had two nuclei (Fig. 10, *a*) and it was selected with the idea that cell division might follow. Instead of that, in 18 minutes the two nuclei touched, and then without any loss of nuclear

[4] These observations confirm and extend those of Rosenthal (Rosenthal, N., *Arch. Int. Med.*, 1917, xx, 184).

TABLE VI.

Correlation between Counts Made with Vital and Oxidase Stains.

	Vital counts.					Oxidase counts.				
	Counts.....	1	2	3	4	Counts.....	1	2	3	4
		per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*
Dec. 15	Myelocytes, Type A.....	13	5			Cells with one granule.....	16	18	19	25
	" " B.....	14	29			" " scattered granules.....	20	8	6	17
	" " C and polymorpho-					" " massive "	22	25	27	21
	nuclear neutrophils.....	19	17			Damaged cells.....	3	2	4	0
	Unclassified.....	0	21				—	—	—	—
		—	—			Total.....	61	53	56	63
	Total.....	46	72							
" 17	Myelocytes, Type A.....	4	6			Cells with one granule.....	8	9	3	
	" " B.....	17	10			" " scattered granules.....	6	9	17	
	" " C and polymorpho-					" " massive "	32	30	30	
	nuclear neutrophils.....	35	46			Damaged cells.....	10 (42)	7 (37)	5 (35)	
	Unclassified.....	0	0				—	—	—	
		—	—			Total.....	56	55	55	
	Total.....	56	62							
" 18	Myelocytes, Type A.....	29				Cells with one granule.....	5 (46)	12 (46)		
	" " B.....	21 (50)				" " scattered granules.....	41	34		
	" " C and polymorpho-					" " massive "	23	21		
	nuclear neutrophils.....	18				Damaged cells.....	0	0		
	Unclassified.....	0					—	—		
		—				Total.....	69	67		
	Total.....	68								

Dec. 19	Myelocytes, Type A	1	1	0	2	Cells with one granule	2	5		
	" " B	41	22	27	31	" " scattered granules	32	36		
	" " C and polymorphonuclear neutrophils	16	33	29	25	" " massive "	24	13		
	Unclassified	0	0	0	0	Damaged cells	2	6		
		—	—	—	—		—	—		
	Total	58	56	56	58	Total	60	60		

membrane except at the point of contact a bridge was formed as is shown in Fig. 10, *b*. This process was apparently similar to the way in which two oil drops flow together. 5 minutes later the bridge began to narrow again so that the process did not go to a complete fusion of the two nuclei as in the cell of Figs. 9, *a* and 9, *b*, but after an hour the two nuclei separated completely so that the cell again looked as it did when first drawn. Complete division of the nucleus, not followed by cell division, is shown in the case of two myelocytes of Type B in Figs. 6 and 7. In the first cell, Fig. 6, the nucleus underwent complete division from the stage shown in the drawing in 6 minutes; the two nuclei remained separate for 10 minutes and then rejoined and returned to the original condition in 5 more minutes. This was a typical myelocyte, Type B, in which the neutrophilic granules were moving in the cytoplasm. The other cell, Fig. 7, showed a condition which we noted several times; namely, one in which there were irregular and uneven threads of chromatic material in the bridge of the nucleus. Instead of dividing equally these threads became unevenly distributed between the two daughter nuclei. In the material at autopsy we have also found this same appearance in some of the constricted nuclei; we concluded that some of the examples of amitosis were abnormal in showing an atypical formation of chromosomes. In another paper we (6) are giving our concept of normal amitosis. This point raises the question as to whether the appearance of these incomplete mitotic figures represents an abortive attempt at mitotic division, or a very abnormal type of amitotic division. The last drawing of amitosis is shown in Fig. 11, selected to show that the small cells could also divide. The special interest in this cell was that the first division, which was watched through to completion, was an even division of the nucleus, but while this division was taking place, one of the two resulting nuclei also started to divide at the point marked *x*, and when this division had taken place there were three unequal nuclei.

On Dec. 6, all of the cells showing amitosis were myeloblasts; out of the 21 per cent of the cells which were found dividing 1 per cent were small cells, 3 per cent were of intermediate size, and 17 per cent were large myeloblasts. Up to Dec. 6, all of the cells in amitosis were myeloblasts. From that time on both myeloblasts and myelocytes were found dividing but there were more examples of division among the myeloblasts, until Dec. 7, when twenty-three myelocytes and only two myeloblasts were found in division. We followed many other examples of amitosis which were not drawn. In one instance, studied on Dec. 6, we found a nucleus with four indentations and finally one part of the nucleus separated off entirely, leaving a large three lobed nucleus and a small oval one. We also found cells with two nuclei in which the larger mass seemed to be splitting into four parts, which resulted in a final division of the nucleus into five separate parts. Again we found cells with two nuclei in which the smaller had a diameter less than a fifth of the larger one.

On Dec. 10, a study was made on a slide which had no stain in order to test whether the toxicity of the Janus green was producing the nuclear changes. In the count of 100 cells five myeloblasts and two myelocytes were found undergoing amitosis. One of the myeloblasts, when first seen, had a nucleus which was evenly constricted as if in preparation for division; this division was completed and then one of the nuclei again divided asymmetrically, so that the resulting cell had three nuclei, two of them approximately equal and one smaller. This process took 37 minutes.

In one instance studied on Dec. 15, an example of amitosis was followed in a red blood cell. In this instance the nucleus divided and there were three abortive attempts at cell division in three entirely different planes, the nuclei having shifted their position in the cell so that each time the cytoplasmic plane was between the two nuclei. One of the cytoplasmic cleavages was almost complete; once, one of the nuclei approached the edge of the cell and caused it to bulge as if this nucleus was going to be extruded; this nucleus, however, returned to the center of the cell, which eventually rounded up and became quiescent.

TABLE VII.

Percentage of Amitosis.

Nov. 27	Dec. 5	Dec. 5	Dec. 5	Dec. 6	Dec. 10	Dec. 12	Dec. 13	Dec. 14	Dec. 15	Dec. 17	Dec. 18	Dec. 19
	10 a. m.	*1 p. m.*	*2 p. m.*									
2	10	8	21	21	7	12	7	7.5	8	25	15	13

In following the records of the percentage of amitosis seen throughout the case we have included both cells in which the nuclei were actually seen dividing and cells with two or more nuclei.

Our records are shown in Table VII.

These figures are shown graphically on Chart 2, in comparison with the curves of the myelocytes, Type C, and of the myelocytes, Types A and B, taken together. The most striking point is that the times at which the highest percentages of amitoses were reached were after the transfusions. The count on Dec. 5, made 2 hours after the transfusion showed an increase to 21 per cent which was maintained on the morning of Dec. 6; the second peak was on Dec. 17, reaching a maximum of 25 per cent, apparently a somewhat delayed reaction when contrasted with the former post-transfusion increase.

The process of amitosis was extremely varied and peculiar in many of the cells observed in this case. There were markedly uneven divisions of the nuclei with immediate, or delayed cell division, the fusion of two nuclei after complete separation, and the presence of threads of

chromatin in the zone of cleavage of some of the dividing nuclei. Such peculiarities in the process of amitotic division suggest strongly that the process in this case was a distinctly abnormal one.

DISCUSSION.

From the study of these cases of myeloblastic leucemia it has seemed possible to discriminate the myeloblast with the vital technique, and to follow the steps of the maturation of the myelocytes. We consider that the *primitive white blood cell* (1), from which the leucocytes, monocytes, and lymphocytes are derived, is a cell with a large nucleus in proportion to its cytoplasm, and containing a few unevenly distributed rod-shaped mitochondria. The especial characteristic of the myeloblast is the great increase in the number, and decrease in the size of the mitochondria; *i.e.*, the primitive cell becomes a myeloblast by the development of many small mitochondria in the cytoplasm. Thus, in our concept, an increase of mitochondria in developing blood cells is correlated with the phase of maturation of a *primitive white cell* into a myeloblast, while on the other hand the maturation of the myeloblast through the stages of the myelocyte into the leucocyte is correlated with a decrease in the number of mitochondria. These observations, on living cells, represent only one factor in the change in the cytoplasm; another factor, the degree of the reaction to basophilic dyes in fixed smears, has long been known. In our studies of the marrow in rabbits we have followed the changes of the mitochondria from the primitive cell toward the myeloblast but in this case there were no cells more primitve than the myeloblast. We speak of the myeloblast as a level or a stage in the development of the leucocyte, and it was obviously at this level or with this type of cell that the pathological involvement in this case occurred.

Considerable variations in the exact distribution of the mitochondria of the myeloblasts are shown in our Figs. 1, 3, 5, 9, *a*, 10, *a*, and 11. In general, we consider the distribution shown in Figs. 3 and 5 as the typical arrangement; here the nucleus is eccentric and the great mass of the granules is in the larger zone of the cytoplasm. In the cell of Fig. 3, the nucleus was more centrally placed, while in the stages of the myelocytes of Type B as shown in Figs. 6 and 7 there was an approximately equal distribution of stainable substances on the two sides of

the nucleus, these cells being at a stage when the granules were moving in the cytoplasm. Another variation in which the mitochondria are limited to one side of the nucleus is shown in Figs. 9, *a* and 9, *b*. We feel confident that the development of a large content of mitochondria characterizes the myeloblast, and is a condition which precedes the differentiation of neutrophilic granules in the cytoplasm.

The variation in the size of the myeloblasts from a cell whose diameter is more than twice that of a red blood cell to one even smaller we think correlated with rapid cell division, since immediately after division the resulting cells must be half the size of the original cell. In general we found more large cells undergoing division, but the fact that we found cells of all sizes, even so small as shown in Fig. 11, undergoing division indicated that there was some disturbance between the normal alternation of the phases of growth and division.

The cell which we call the myeloblast is at least one of the types of cells called by Pappenheim (4) the *Lymphoidocyte*. We consider that he also included in the term *Lymphoidocyte*, types that we would discriminate as monoblasts and lymphoblasts. In his Atlas, on page 84, Pappenheim postulated a still more primitive cell than his *Lymphoidocyte*, a form from which all of the blood cells must be derived.

We think that our studies (1) lead to an identification of the primitive cell which Pappenheim postulated, and that it is the forerunner of all three types of the white blood corpuscles. With the method of depletion of the bone marrow this cell can be found at will, so it is no longer a hypothetical type. The study of this case has brought out a factor fundamental to the identification of the myeloblast, the definite forerunner of the leucocyte. This factor is the development of large numbers of tiny mitochondria, which gradually disappear as the cell becomes the mature leucocyte. In the maturation of the myeloblast into the myelocyte, there can be discriminated three phases, whose correlation with the three types of the oxidase reaction is close enough to indicate that such a grouping is not accidental. We think that the significance in recognizing these three types consists in the value of knowing how primitive the type of myelocyte is which may appear in the circulating blood. For example we have found the myelocyte of Type B in the circulation in the case of experimentally infected rabbits.

There are three degrees of the reaction to neutral red of the neutrophilic granules in human blood; the original phase is found in the A and B types of myelocytes, in which the granules react more intensely to neutral red than in the stage of the leucocyte; the second stage is that of the myelocyte, Type C, and the leucocyte, which show a reaction which is clear but not intense; and the third phase is that of the *non-motile* leucocyte (5), in which the neutrophilic granules have become swollen, more refractive, and take no vital dye whatever. After the first transfusion we found a few myelocytes which showed the non-motile phase of the neutrophilic granule.

In regard to motility, myeloblasts and myelocytes show no ameboid movements. Individual granules may move in the cytoplasm, the cell itself may change its position slowly, but in these early forms there is none of that active streaming of cytoplasm, correlated with rapid movement of the cell, which characterizes the leucocyte up to the time of its death. In our preparations the leucocytes are always in ameboid activity under normal conditions of temperature, except during the first few seconds after making the preparations, and we are inferring that this is probably the case when these cells are in the tissues. In regard to the effects of the transfusions the matter is much too complex with our present state of knowledge to analyze, but three points of view may be presented; first, that the blood introduced contained a stimulant which caused the myeloblasts to become myelocytes; second, that the donor's blood cells supplied, for a brief interval, substances used by the tissues and thus relieved the demand on the blood-forming tissues; or third, that the new blood temporarily counteracted substances that were checking the maturation of the myeloblasts. Such a statement shows how completely we lack a knowledge of the forces that produce the maturation of the blood cells and the stimuli which bring them into the circulation.

The amitoses were the most striking feature of this case. In our vital counts we discriminated between the cells in which we actually saw the nuclei in the process of division and those in which we found two, three, or four nuclei, but in the percentage of amitosis we have included both types of nuclear changes. The marked variety in these phenomena, the uneven divisions of the nuclei, the usual delay in cell division following these nuclear divisions, the examples of abortive

attempts at nuclear division, as shown in many cases in which nuclei divided and reunited, the example of abortive cell division in which three different planes were tried and then abandoned, and the presence in some and the absence in others of atypical chromosomes make us regard the amitosis as evidence of a disordered cellular process. We are the more sure of this since, in following the type of amitotic cell division of normal cells in our vital preparations, we have found an orderly and characteristic sequence of events (6). We do not consider that this case was wholly unusual in the fact of amitosis though the amount of it may have been in excess of the average case.

CONCLUSIONS.

1. Myeloblasts can be discriminated in the supravital technique by the great numbers of tiny mitochondria in the cytoplasm and the absence of any other vitally stainable substance.

2. There are three stages in the maturation of myelocytes.

3. These three phases can be correlated with three types of the oxidase reaction.

4. One case of myeloblastic leucemia showed such an amount of an abnormal type of amitosis as to suggest the disordered cell division of neoplasms.

5. In this case transfusions were correlated with a maturation of myeloblasts into myelocytes, with an increase of the oxidase reaction, and with an increase in amitosis.

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EXPLANATION OF PLATES.

PLATE 39.

The cells on this plate were drawn from living specimens which had been supravitally stained with neutral red and Janus green. The magnification is about 3,300; a red corpuscle is given for comparison. Figs. 3 to 6 were from Case C. M., Hebrew Hospital, Baltimore.

FIG. 1. Myeloblast from the blood of a case of myeloblastic leucemia (B. R., Johns Hopkins Hospital, Med. No. 50043, Path. No. 7716). The nucleus of this cell showed a diffuse chromatin network and was slightly eccentric. In the cytoplasm there were tiny mitochondria.

FIG. 2. Myelocyte, Type A, from a case of myeloid leucemia (J. F. F., Johns Hopkins Hospital, Med. No. 50256). The chromatin network of the nucleus was dense; the small clump of granules stained in neutral red represents the first neutrophilic granules or their precursors and indicates that this cell belongs to Type A.

FIG. 3. Myeloblast from the case of myeloblastic leucemia, drawn Dec. 5, 2 hours after the first transfusion. The nucleus had a coarse chromatin network and three nucleoli. The cytoplasm contained great numbers of tiny mitochondria.

FIG. 4. Myelocyte, Type A, drawn Dec. 17. The nucleus had a dense chromatin network and one nucleolus. The cytoplasm contained many tiny mitochondria and a small clump of granules that stained in neutral red.

FIG. 5. Myeloblast, Type A, drawn Dec. 17. The nucleus showed a massive chromatin network without definite nucleoli. There was a clump of four neutrophilic granules on one side of the nucleus and three on the other.

FIG. 6. Myelocyte, Type B, drawn Dec. 18. When the drawing was started the cell was as shown in this figure; in 6 minutes the nuclear division was completed, after 10 minutes the nuclei started to rejoin each other, and in 5 minutes the nucleus had returned to its original condition. The granules in the cytoplasm were moving.

PLATE 40.

All the cells on this plate were from the case of myeloblastic leucemia (C.M., Hebrew Hospital, Baltimore). They were drawn from living cells, supravitally stained with neutral red and Janus green. Figs. 7, 8, 9, *a*, and 9, *b*, were drawn at a magnification of about 3,300. The cells of Figs. 10, *a*, 10, *b*, and 11 were drawn at a magnification of about 5,700 and a red blood corpuscle is given for comparison.

FIG. 7. Myelocyte, Type B, drawn Dec. 18. This cell showed atypical threads of chromatin in the line of cleavage of the nucleus. The nucleus of this cell divided completely in 32 minutes.

FIG. 8. Myelocyte, Type C, drawn Dec. 5, 2 hours after the first transfusion. The neutrophilic granulation was massive and the mitochondria were reduced to a few granules in the periphery.

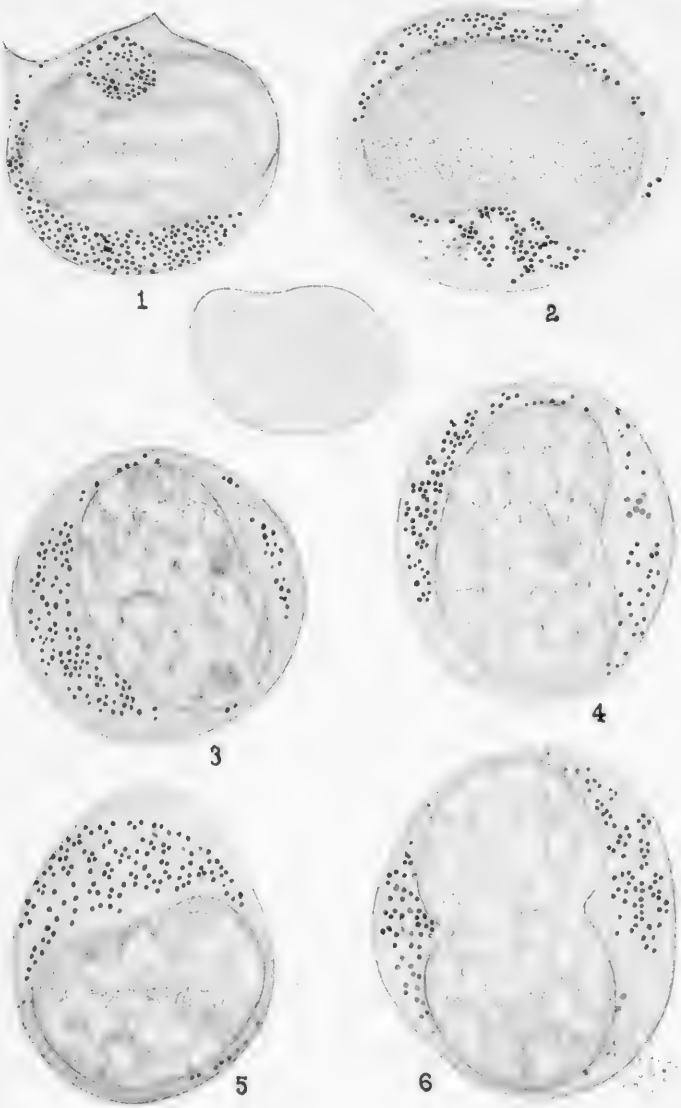
FIG. 9, *a*. Myeloblast, drawn Dec. 4, to show amitosis. When first seen the cell had three nuclei, the two smaller ones being widely separated and wholly distinct, and there was no constriction in the nucleus itself. 15 minutes later, the condition was as shown in this figure.

FIG. 9, *b*. The same cell as Fig. 9, *a*. While this cell was being drawn the two small nuclei fused into one and the cell divided. The processes of the fusion of the two small nuclei and the division of the cytoplasm took 25 minutes. During this entire time the cytoplasm was refractive and clear except for the mitochondria, which became redistributed as shown in the drawings.

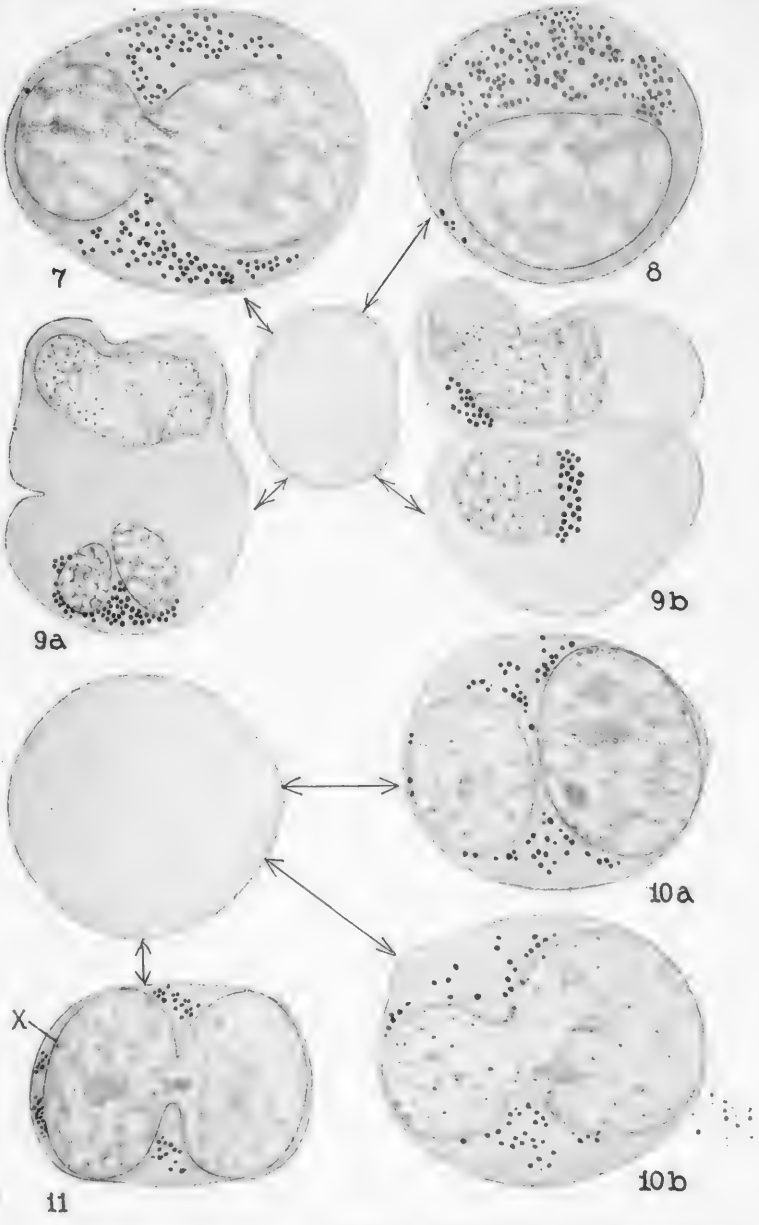
FIG. 10, *a*. Myeloblast of small size, drawn Dec. 5. The cell was from a preparation taken before the transfusion. When first seen the cell had two unequal nuclei, one of which had two or three nucleoli. The cell remained in the condition shown for 18 minutes.

FIG. 10, *b*. The same cell as Fig. 10, *a*. The two nuclei touched at one point and then flowed together as shown in this figure. After remaining in this condition for 5 minutes the bridge between the two nuclei began to narrow and in 52 minutes the nucleus divided completely so that the cell again looked as it did in Fig. 10, *a*.

FIG. 11. Myeloblast, drawn Dec. 5, before the first transfusion. When first seen the cell was as represented here. While it was being drawn there was a complete division of the nucleus in the line of the deep cleft and while this was taking place the segment on the left divided completely in the place indicated by the line marked *x*. This process took 12 minutes and resulted in a cell having three unequal nuclei.



(Sabin *et al.*: Maturation of myeloblasts into myelocytes.)



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